

REPORT FINALE

2°Anno

Il problema emergente delle microplastiche nel Mar Mediterraneo: il potenziale impatto sulla balenottera comune come modello di “descrittore ambientale”



M. Cristina Fossi

*Silvia Casini, Cristina Panti, Letizia Marsili, Ilaria Caliani, Daniele Coppola, Matteo Giannetti,
Matteo Bainsi, Cristiana Guerranti, Tommaso Campani, Roberta Minutoli*

*Università di Siena, Dipartimento di Scienze Fisiche, della Terra e dell’Ambiente, Siena, Italia
fossi@unisi.it*

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PREMESSE

A seguito degli importanti risultati conseguiti dallo svolgimento del primo anno del progetto, in riferimento al riconoscimento dell'importanza di tali indagini sullo scenario internazionale come confermato dal comitato scientifico dell'International Whaling Commission (Panama 2012, Woods Hole 2013, St. Andrews 2014) e come confermato dalla necessità di implementare il Descrittore 10 (Marine Litter) della Direttiva Quadro della Strategia Marina Europea, si è ritenuto essenziale lo sviluppo di un secondo anno di attività del progetto sui seguenti aspetti:

a) Raccolta di un numero superiore di campioni di microplastiche in un'area più vasta del Santuario Pelagos che comprenda le aree del:

- Mar Ligure
- Mar di Sardegna
- Mar Tirreno (Arcipelago Toscano)

b) Individuazione e conteggio delle macroplastiche superficiali in aree di indagine del Santuario Pelagos;

c) Sviluppo di tecniche diagnostiche più specifiche per evidenziare la presenza di microplastiche in individui planctonici e neustonici (rilevamento con tecniche di microscopia ottica e microscopia elettronica a scansione e trasmissione);

d) Campionamento di biopsie cutanee di balenottere comuni *free-ranging* e relative indagini ecotossicologiche nell'area del Santuario Pelagos;

e) sviluppo di nuovi biomarkers su individui *free-ranging* di varie specie di cetacei (oltre alla balenottera comune) specifici per individuare la presenza e gli effetti degli additivi delle plastiche (in particolare ftalati).

1

METODOLOGIE UTILIZZATE



1. METODOLOGIE

A) RACCOLTA E CARATTERIZZAZIONE DI CAMPIONI DI MICROPLASTICHE (SUPERFICIALI E COLONNA D'ACQUA) IN SOTTO-AREE DEL SANTUARIO PELAGOS CHE COMPRENDONO LE ZONE DEL:

- Mar Ligure
- Mar di Sardegna
- Mar Tirreno (Arcipelago Toscano)

Campionamento microplastiche e plancton superficiale

I campionamenti vengono effettuati con retino conico standard WP2 (bocca con anello metallico tondo da 57 cm di diametro, lunghezza 2,60 m circa, vuoto di maglia da 200 μm) equipaggiato con flussimetro per la stima dei metri cubi di acqua filtrata.

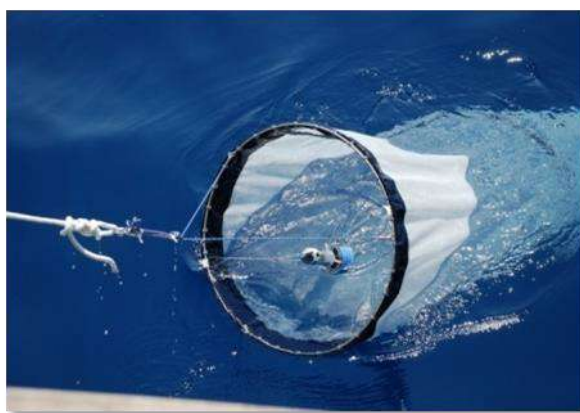


Fig.1. Retino conico standard WP2 con flussimetro.

Metodologia di prelievo

Il campionamento viene eseguito con imbarcazione in movimento: il retino è calato sotto la superficie dell'acqua e viene trainato alla velocità di 1,5 nodi per 15 minuti. Il retino una volta salpato, deve essere sciacquato dalla bocca verso il bicchiere, con getto d'acqua salata a pressione al fine di convogliare il materiale verso la parte terminale del retino (bicchiere di raccolta).

Tempo necessario per ogni retinata: 25-30 minuti

Trattamento campioni a bordo: per ogni retinata il campione raccolto è portato al volume di due litri e suddiviso in due sub-aliquote da 1 litro ciascuna, una filtrata su garza con stesso vuoto di maglia del retino ed immediatamente congelata per successive analisi di accumulo contaminanti, l'altra fissata in acqua di mare e formalina tamponata al 4% per successive analisi qualitative.

Campionamento mesozooplancton verticale

Il campionamento di microplastiche e plancton nella colonna d'acqua è stato eseguito con retino conico standard WP2 (bocca con anello metallico tondo da 57 cm di diametro, lunghezza 2,60 m circa, vuoto di maglia da 200 μm) equipaggiato con flussimetro per la stima dei metri cubi di acqua filtrata.

Metodologia di prelievo: il campionamento viene eseguito con imbarcazione ferma. Il retino viene calato manualmente sino alla profondità di 50 m e recuperato alla velocità di 1m/s. Il retino deve

poi sciacquato dalla bocca verso il bicchiere, con getto d'acqua a pressione al fine di convogliare il materiale verso la fine della rete.

Tempo necessario per ogni retinata: 2-3 minuti

Trattamento campioni a bordo: il campione raccolto è portato al volume di due litri e suddiviso in due sub-aliquote da 1 litro ciascuna, una filtrata su garza con stesso vuoto di maglia del retino ed immediatamente congelata per successive analisi di accumulo contaminanti, l'altra fissata in acqua di mare e formalina tamponata al 4% per successive analisi quali-quantitative.

B) INDIVIDUAZIONE E CONTEGGIO DELLE MACROPLASTICHE SUPERFICIALI NELLE SUDETTE AREE DI INDAGINE DEL SANTUARIO PELAGOS CHE COMPRENDE LE AREE DEL:

- Mar Ligure
- Mar di Sardegna

Seguendo uno specifico piano di campionamento è stato eseguito un monitoraggio qualitativo e quantitativo delle macroplastiche rilevate sulla superficie del mare. I dati verranno successivamente elaborati in termini quantitativi, dimensionali, qualitativi. Di seguito viene riportata la metodologia utilizzata, derivata da un esame critico delle principali metodologie utilizzate a livello internazionale.

Messa a punto del protocollo “Survey e monitoraggio del marine litter in mare”

La metodologia più utilizzata per la quantificazione ed il monitoraggio dei rifiuti marini galleggianti è l'osservazione visiva dalle imbarcazioni. I risultati ottenuti tramite questa metodologia sono fortemente dipendenti dall'obiettivo della campagna di monitoraggio, dall'osservatore, dal protocollo utilizzato, dall'imbarcazione e dalle condizioni meteo-marine di osservazione. Poiché i risultati dipendono da tutti questi diversi fattori esterni, il confronto tra diverse indagini e le valutazioni di tendenza sono difficili da effettuare. L'utilizzazione di protocolli condivisi per la segnalazione e la registrazione dettagliata sia delle condizioni di osservazione, sia degli avvistamenti sono, di conseguenza, molto importanti. Esistono alcune linee guida e pubblicazioni scientifiche che forniscono diversi approcci per la quantificazione dei rifiuti marini e sottolineano le difficoltà di raccolta dei dati e l'applicazione di una metodologia robusta per l'ottenimento di dati universalmente comparabili. Le linee guida UNEP (Cheshire et al., 2009) descrivono come un monitoraggio dettagliato può essere effettuato in aree ristrette (5 x 5 km) attraverso indagini dedicate, oppure le indagini possono essere effettuate lungo transetti. Questa metodologia è utilizzata principalmente quando si utilizzano navi impiegate per scopi diversi e messe a disposizione per questo tipo di monitoraggio. La US National Oceanographic and Atmospheric Administration (NOAA) ha sviluppato un protocollo denominato “Shipboard Observation Form for Floating Marine Debris” (Arthur et al., 2011). Questo protocollo si basa sull'esperienza acquisita dal NOAA nei precedenti studi condotti sui rifiuti galleggianti marini. L'obiettivo di questo metodo è quello di essere in grado di calcolare la densità dei rifiuti marini all'interno dell'area attraversata dal transetto, utilizzando una versione leggermente modificata della formula usata da Matsumura e Nasu (1997), Shiimoto e Kameda (2005) e Thiel et al. (2003).

L'identificazione e la classificazione corretta degli oggetti galleggianti può essere difficile, per questo, sono stati proposti sistemi di classificazione semplificati. Il sistema di classificazione operato dovrebbe essere compatibile con quello riportato nel Descrittore 10 della EU MSFD (Task Group D10, EUR 25009 EN – 2011).

Nell'ambito del presente progetto è stato effettuato un monitoraggio dei rifiuti galleggianti nelle aree del Santuario Pelagos sfruttando sia le imbarcazioni utilizzate per la raccolta delle biopsie cutanee di cetacei e campioni di plancton e microplastiche, sia navi di opportunità che seguivano transetti predefiniti. L'osservazione è stata effettuata a prua dell'imbarcazione (ove possibile) con le spalle rivolte al sole e solamente in condizioni meteo-marine ottimali (mare calmo e visibilità buona). Sono state presi in considerazione i rifiuti galleggianti osservati entro i 20 metri dall'imbarcazione, poiché ad una distanza maggiore gli oggetti sono difficilmente classificabili. L'avvistamento, senza l'utilizzo del binocolo, è stato effettuato cambiando ogni 30 minuti l'osservatore per evitare l'affaticamento dell'operatore. La scheda utilizzata per la raccolta dei dati (vedi Tab.1) è stata ripresa e modificata dal protocollo NOAA (NOAA Form 57-11-14 (6-12)). All'inizio del turno di avvistamento l'operatore ha compilato la prima parte della scheda (data, orario, coordinate, ecc.) e sono state annotate le condizioni meteo-marine nelle apposite caselle. Se durante il monitoraggio sono state osservati cambiamenti nelle condizioni meteo-marine, sono state annotate nell'apposita casella. Per ogni oggetto galleggiante (dimensioni superiori a 2,5 cm) è stata posta una crocetta all'interno della categoria di appartenenza. Alla fine dell'attività di avvistamento sono state annotate orario e coordinate.

Poiché lo scopo del progetto prevedeva solo il conteggio di macroplastiche, è stata effettuata successivamente un'elaborazione per il calcolo dei rifiuti galleggianti, tenendo in considerazione solo gli oggetti definiti come “plastiche” nella scheda.



Fig.2. Macroplastiche galleggianti campionate con evidenti eventi di *fouling*.

C) SVILUPPO DI TECNICHE DIAGNOSTICHE PIÙ SPECIFICHE PER EVIDENZIARE LA PRESENZA DI MICROPLASTICHE IN INDIVIDUI PLANCTONICI (RILEVAMENTO CON TECNICHE DI MICROSCOPIA OTTICA E MICROSCOPIA ELETTRONICA A SCANSIONE E TRASMISSIONE)

Questo indagine è stata applicata su vari organismi planctonici al fine di individuare la presenza di microplastiche all'interno degli apparati gastro-intestinali degli organismi. Per quanto riguarda l'utilizzo della microscopia elettronica, sono stati utilizzati due tipi di microscopi, il SEM (Scanning Electron Microscope) ed il TEM (Transmission Electron Microscope). Il primo approccio con il TEM, tramite sezioni ultrasottili, fornirà dati ultrastrutturali sulle particelle delle microplastiche e la loro localizzazione tissutale. Il secondo approccio, attraverso l'utilizzo del SEM (visione dei campioni a risoluzione più bassa) servirà, mediante rilevatori di elettroni retrodiffusi, a calcolare le quantità relative di queste particelle nei tessuti scelti come target. La principale difficoltà di questo tipo di indagine sarà quella di mettere a punto una metodologia ed un protocollo per la scelta del tessuto target e la preparazione del campione sul quale poi indagare la presenza delle microplastiche. Fino ad oggi, i pochi studi presenti su specie marine riguardano principalmente invertebrati filtratori quali i bivalvi nei quali è stata notata, tramite microscopia elettronica, la presenza di microplastiche, tuttavia mancano dati sugli organismi planctonici che risultano di maggiore interesse in ambiente pelagico.

D) CAMPIONAMENTO DI BIOPSIE CUTANEE DI BALENOTTERE COMUNI (ED ALTRE SPECIE DI CETACEI) FREE-RANGING E RELATIVE INDAGINI ECOTOSSICOLOGICHE NELL'AREA DEL SANTUARIO PELAGOS

Le biopsie cutanee di cetacei sono un materiale biologico particolarmente idoneo per la stima del rischio ecotossicologico di cetacei *free-ranging* (Fossi *et al.*, 2010). In questo progetto vengono utilizzate biopsie cutanee come strumento per una diagnosi della presenza e degli effetti dei derivati delle microplastiche (es. ftalati) e contaminanti persistenti da esse veicolati (POPs - OCs) nella popolazione mediterranea di balenottera comune ed in altre specie di cetacei (odontoceti).

Tecnica di campionamento della biopsia cutanea: campioni di epidermide, derma e grasso sottocutaneo sono stati ottenuti da esemplari di *B. physalus free-ranging* usando una balestra e dardi modificati con una punta da biopsia in alluminio (0.9 cm di diametro, 4.0 cm di lunghezza) e un galleggiante. E' stata utilizzata una balestra Barnett Wildcat (150 libbre). Per evitare la possibilità di trasmissione di infezioni batteriche o virali tra diversi animali e cross-contaminazione dei campioni, la punta da biopsia è stata sterilizzata tutte le volte prima di effettuare il campionamento. E' stata prestata particolare attenzione a prelevare i campioni di biopsia in prossimità della pinna dorsale e sempre nella stessa parte del corpo dell'animale. La procedura di

avvicinamento all'animale consiste nell'approcciare l'esemplare ad una velocità da bassa a moderata e sparare il dardo ad una distanza di circa 20-50 m. La biopsia cutanea viene posta immediatamente in azoto liquido o in mezzo per colture organotipiche (CITES Nat. IT025IS, Int. CITES IT 007).



Fig.3. Campionamento di biopsie di balenottera comune durante le campagne nel Santuario Pelagos.

E) INDAGINI ECOTOSSICOLOGICHE SU BIOPSIE CUTANEE E CAMPIONI DI PLANCTON PRELEVATI NELL'AREA DEL SANTUARIO PELAGOS

Valutazione dei livelli dei contaminanti di natura antropica: la stima dei livelli dei contaminanti nella specie target rappresentano un importante parametro di esposizione; questi dati possono essere correlati ai dati provenienti dalle risposte dei biomarker per una valutazione completa dello stress tossicologico della specie. Dato che i cetacei accumulano alte concentrazioni di composti tossici come ftalati, bisfenolo A, idrocarburi policiclici aromatici (IPA), composti organoclorurati (OC) e ritardanti di fiamma brominati (BFR), alcuni di questi contaminanti sono stati analizzati nei campioni di biopsia cutanea dei cetacei e nei campioni di plancton attraverso specifiche metodologie analitiche ottimizzate per le diverse matrici biologiche.

Marcatori diagnostici per l'esposizione ai contaminanti: le isoforme del citocromo P450 (CYP1A/CYP2B) e il recettore per i composti arilici (Aryl Hydrocarbon Recetor – AHR) sono marcatori di esposizione ai contaminati di natura antropica come IPA, OC, BFR. L'AHR ha un ruolo fondamentale nella regolazione della trascrizione dell'mRNA delle isoforme del CYP in risposta al legame del recettore con gli IPA, composti alogenati aromatici e composti diossino-simili. I loro livelli di espressione (sia genica che proteica) sono stati analizzati nelle biopsie cutanee mediante PCR Real Time quantitativa (qRT-PCR) e Western Blot (WB).

Marcatori diagnostici per alterazioni nella riproduzione: molti dei meccanismi legati ad eventi di distruzione endocrina sono mediati da recettori (per esempio la modulazione degli steroidi avviene attraverso i recettori per gli estrogeni/androgeni o tramite *cross-talk* dei recettori con l'AHR). È quindi estremamente importante studiare nell'ambito di questo progetto la modulazione dell'espressione di tali recettori in risposta ai contaminanti derivati dalle microplastiche della quale è nota la potenzialità di distruttori endocrini (ftalati, bisfenolo A, ritardanti di fiamma). I marcatori di alterazione delle capacità riproduttive (recettore per gli estrogeni – ER) sono stati analizzati mediante qRT-PCR.

Marcatori diagnostici di stress generale: la pelle dei cetacei è esposta ad un insieme di stress ambientali e antropici; biomarker di stress generali sono quindi essenziali per definire lo stato di salute generale dell'organismo. La perossidazione lipidica comprende una serie di reazioni a catena da parte dei ROS dovuta ai loro doppi legami ed è il risultato delle interazioni dei radicali lipidici e/o della formazione di specie non radicaliche da radicali lipidici perossidati. La maggior parte dei prodotti di questo processo sono tossici e mutageni attivi. La perossidazione lipidica è stata valutata nelle biopsie cutanee misurando i livelli di MDA (malondialdeide) mediante test spettrofotometrici.

Realizzazione di esperimenti *ex-vivo*: è stata indagata la sensibilità e specificità dello strumento diagnostico multidisciplinare (descritto precedentemente) in colture organotipiche di biopsie da esemplari *free-ranging* di balenottera comune e di altre specie di cetacei esposte a concentrazioni crescenti di miscele di ftalati e bisfenolo A.

2

AREE ED ATTIVITA' DI CAMPIONAMENTO



2. AREE ED ATTIVITA' DI CAMPIONAMENTO

Nell'ambito del 2° anno di attività del progetto sono state svolte 4 campagne di campionamento con tempistiche diverse ed in varie aree del Santuario Pelagos:

- ***1° Campagna – Mar Tirreno (Arcipelago Toscano)***
- ***2° Campagna – Mar di Sardegna***
- ***3° Campagna – Mar Ligure***
- ***4° Campagna – Mar Tirreno (Arcipelago Toscano)***

Le attività di campionamento comprendevano:

- ***Campionamenti di plancton e microplastiche superficiali***
- ***Campionamenti di plancton e microplastiche nella colonna d'acqua***
- ***Campionamenti di biopsie cutanee in esemplari di balenottera comune free-ranging***
- ***Campionamenti di biopsie cutanee in altre specie di cetacei free-ranging***
- ***Monitoraggio macroplastiche***
-

1° Campagna – Mar Tirreno (Arcipelago Toscano)

La prima campagna (parte terminale del primo anno del progetto), della durata di 4 giorni, si è svolta nell'area del Mar Tirreno (Arcipelago Toscano). Lo scopo principale di questa campagna è stato quello di realizzare la seguente tipologia di campionamento:

Campionamenti di plancton e microplastiche superficiali

Di seguito vengono riportati in dettaglio le tempistiche della prima campagna, l'area percorsa, le miglia percorse, il numero e la localizzazione dei campionamenti di plancton e microplastiche effettuati, il numero e la localizzazione degli avvistamenti di esemplari di cetacei ed il numero e la localizzazione delle biopsie effettuate.

Inizio attività: 11 Dicembre 2012 (ore 09:00)
Fine attività: 13 Dicembre 2012 (ore 16:00)
Miglia percorse: 109 miglia percorse di cui on-effort 97,90
Campionamenti di plancton e microplastiche: 8 retinate superficiali

Tab. 2. Retinate nel Mar Tirreno (Arcipelago Toscano)(Dicembre 2012).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|----------------------|--------------|---------------|-----------------------|
| Retinata plancton | Superficiale | MPM46 | N42 27.516 E11 05.840 |
| Retinata plancton | Superficiale | MPM47 | N42 25.066 E11 00.443 |
| Retinata plancton | Superficiale | MPM48 | N42 22.611 E10 55.509 |
| Retinata plancton | Superficiale | MPM49 | N42 22.611 E10 55.509 |
| Retinata plancton | Superficiale | MPM50 | N42 15.428 E11 05.111 |
| Retinata plancton | Superficiale | MPM51 | N42 22.795 E11 19.418 |
| Retinata plancton | Superficiale | MPM52 | N42 22.265 E11 15.422 |
| Retinata plancton | Superficiale | MPM53 | N42 22.706 E11 05.589 |

Di seguito viene riportata la restituzione grafica mediante mappa dedicata dei campionamenti di plancton e microplastiche superficiali svoltisi nella campagna del Dicembre 2012 nel Mar Tirreno (Fig.4).

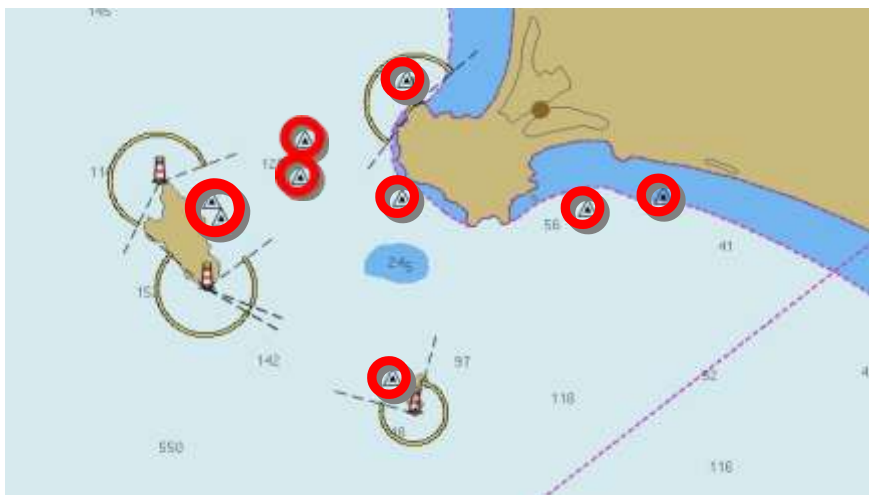


Fig.4. Restituzione grafica mediante mappa dedica dei campionamenti di plancton e microplastiche superficiali Mar Tirreno (Dicembre 2012).

Viste le pessime condizioni meteo marine riscontrate nel periodo della prima campagna di campionamento in Mar Tirreno (Dicembre 2012) si è realizzato un'ulteriore campagna di campionamento nell'area del Mar Tirreno (4° campagna, Novembre 2013) della quale i dettagli sono stati riportati successivamente.

Campionamento di biopsie cutanee nella specie target (Balenottera comune)

Viste le pessime condizioni meteo marine riscontrate nel periodo della prima campagna di campionamento in Mar Tirreno (Dicembre 2012) non si era potuto realizzare nessun avvistamento e campionamento della specie target ne' di altre specie di interesse.

In data 14/10/2013 a seguito di una segnalazione della Capitaneria di Porto di Portoferraio della presenza nella rada di due esemplari di balenottera (presumibilmente balenottera comune), 4 ricercatori dell'Università di Siena si sono tempestivamente recati nella zona di segnalazione dopo una consultazione con il personale della Divisione IV del MATTM. Dopo non aver riscontrato la presenza dei due esemplari nella zona di segnalazione, i ricercatori dell'Università di Siena hanno realizzato, con l'appoggio della Capitaneria di Porto, un *survey* dell'area esterna alla rada di Portoferraio ed hanno, dopo due ore di navigazione, localizzato i due esemplari (uno dei quali presentava estesa parassitosi) e hanno realizzato una biopsia su uno dei due individui (Tab. 3, Fig. 6). Il campione oltre alle analisi ecotossicologiche prefissate, è stato sottoposto anche ad analisi genetica per l'identificazione della specie tramite l'analisi del DNA mitocondriale.

Il gene *cytochrome c oxidase subunit I* (COXI) è stata isolato e sequenziato nel campione da determinare e contemporaneamente in altri due esemplari del genere *Balaenoptera* la cui specie era nota. In particolare, un esemplare di *B. physalus* e un esemplare di *B. bridey* (in quanto quest'ultima specie *sister group* della *B. borealis*). Sebbene l'animale campionato avesse presentato caratteristiche comportamentali e morfologiche atipiche per la balenottera comune, le analisi hanno evidenziato un'omologia della sequenza del 100% con la specie *B. physalus* (Fig. 5).

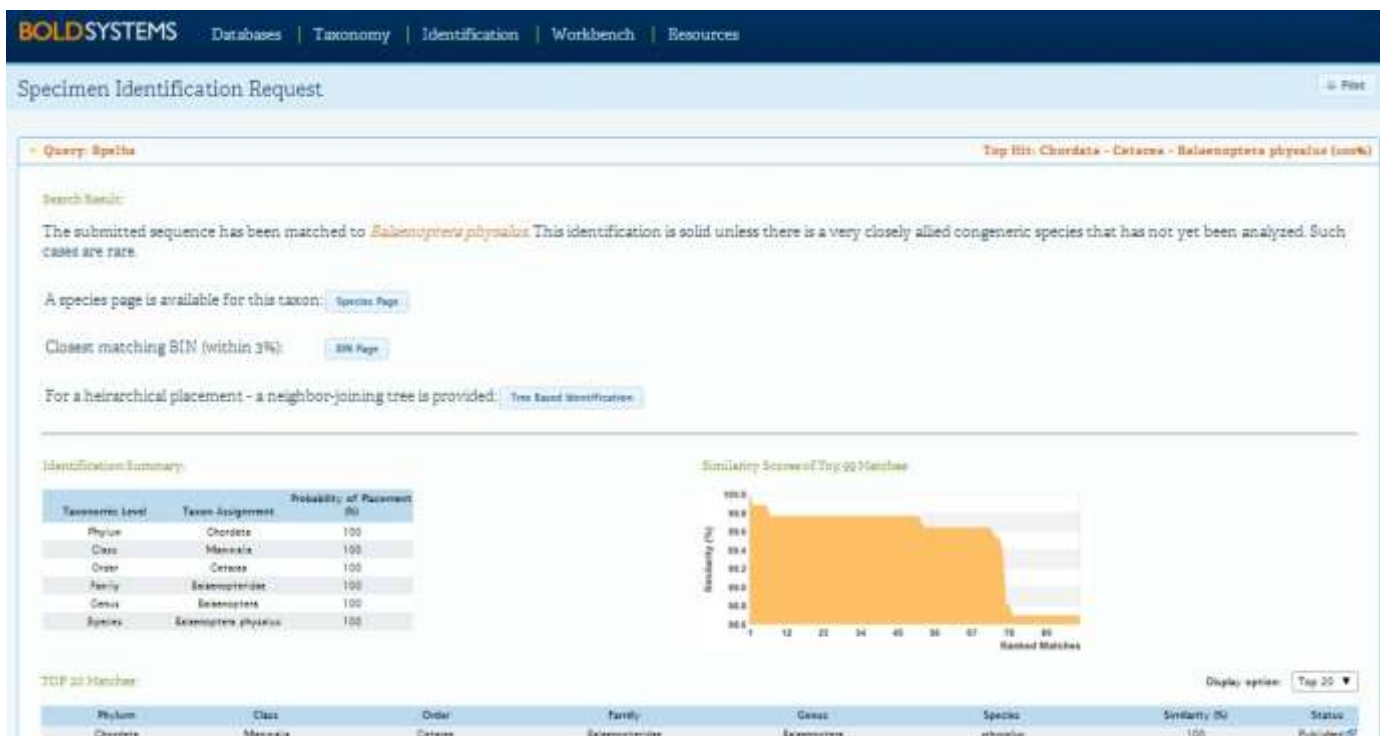


Fig.5. Risultato dell'identificazione della specie campionata in Mar Tirreno (Isola d'Elba, 14 ottobre 2013) mediante il database BOLD (<http://www.boldsystems.org/>).

Tab. 3. Avvistamento di cetacei e biopsie cutanee effettuati all'Isola d'Elba.

| SPECIE | AVVISTAMENTI | BIOPSIE | DATA | COORDINATE |
|------------------------------|--------------|---------|------------|-----------------------|
| <i>Balaenoptera physalus</i> | 2 | 1 | 14/10/2013 | N42 53.007 E10 13.745 |





Fig.6. Restituzione grafica mediante mappa dedicata degli avvistamenti e biopsie cutanee di cetacei effettuate in Mar Tirreno (Isola d'Elba, 14 ottobre 2013). Immagine di uno degli esemplari avvistati.

2° Campagna - Mar di Sardegna

La seconda campagna, della durata di 10 giorni, si è svolta nell'area del Mar di Sardegna. Lo scopo principale di questa campagna è stata quella di realizzare cinque attività diverse:

- ***Campionamenti di plancton e microplastiche superficiali***
- ***Campionamenti di plancton e microplastiche nella colonna d'acqua***
- ***Campionamenti di biopsie cutanee in cetacei free-ranging***
- ***Campionamenti di eufasiacei***
- ***Monitoraggio delle macroplastiche***

Di seguito vengono riportati in dettaglio le tempistiche della campagna, l'area percorsa, le miglia percorse, il numero e la localizzazione dei campionamenti di plancton e microplastiche effettuati, il numero e la localizzazione degli avvistamenti di esemplari di cetacei ed il numero e la localizzazione delle biopsie effettuate.

Inizio attività: 08 Luglio 2013 (ore 11:30)

Fine attività: 17 Luglio 2013 (ore 12:00)

Miglia percorse: 492.88 miglia percorse di cui on-effort 440.72

Campionamenti di plancton e microplastiche: 14 retinate superficiali e 2 retinate notturne

Avvistamenti di cetacei: n° 8 (gruppi) *Stenella coeruleoalba*, n° 1 *Balaenoptera physalus*, n° 5 (gruppi) *Tursiops truncatus*, n° 2 *Grampus griseus* e n° 2 (gruppi) *Delphinus delphis*

Campionamenti effettuati: 32 biopsie *Stenella coeruleoalba*, 1 biopsia *Balaenoptera physalus*, 2 biopsie *Tursiops truncatus*, 1 biopsia *Grampus griseus* e 4 biopsie *Delphinus delphis*

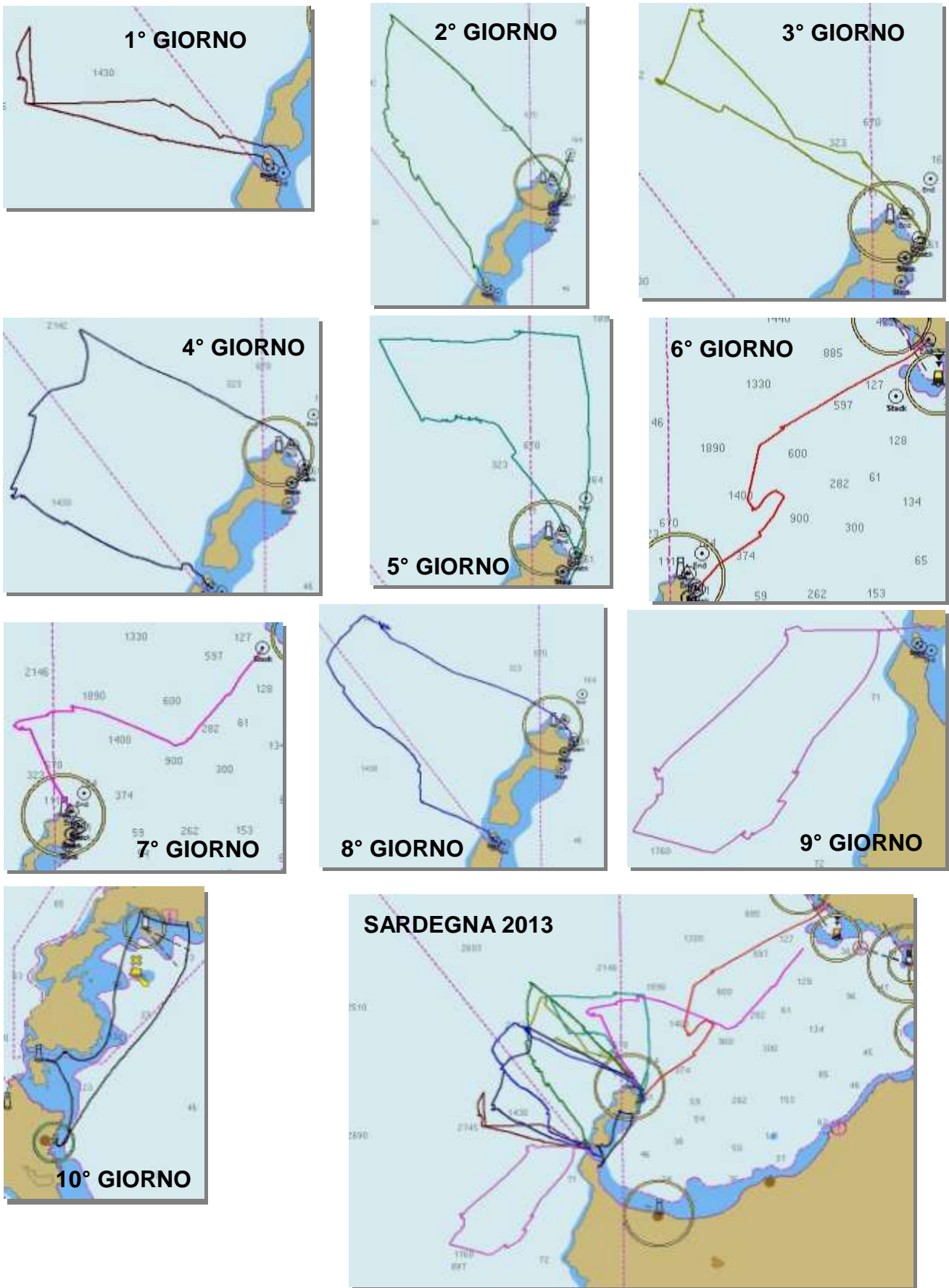


Fig.7. Rotte effettuate durante la campagna svolta nel Mar di Sardegna (Luglio 2013) durante le quali è stato effettuato il survey e monitoraggio delle macroplastiche.

Campionamenti di plancton e microplastiche superficiali e campionamenti nella colonna d'acqua

Durante la campagna di campionamento di Luglio 2013 sono stati condotti 15 campionamenti superficiali ed un campionamento nella colonna d'acqua. Visto i numerosi campionamenti sia superficiali che verticali condotti nella stessa area nella campagna del 2012, si è ritenuto più produttivo in questo secondo anno di attività intensificare i campionamenti in altre aree fino al 2012 non esplorate (vedi sopra). Inoltre, come dimostrato da numerosi lavori su questo argomento ed anche dall'unico lavoro nel Mediterraneo da noi realizzato grazie al finanziamento del primo anno (Fossi et al. 2012), la presenza di microplastiche è rilevante nei primi centimetri superficiali ed estremamente rarefatta nella colonna d'acqua, per questo motivo i campionamenti nella colonna d'acqua sono stati in numero inferiore rispetto a quelli superficiali in questa area.

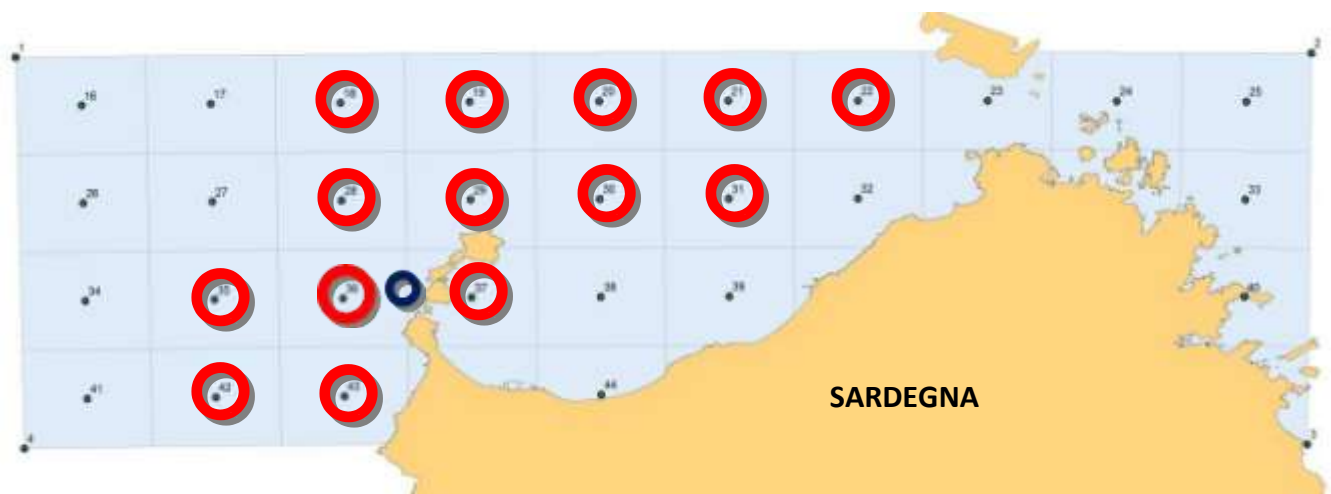


Fig.8. Mappa dei campionamenti di plancton e microplastiche superficiali. In rosso i punti dove sono state effettuate le retinate superficiali di giorno, in blu è evidenziato il punto delle retinate notturne (campionamento eufasiacei).

Tab. 4. Retinate (superficiali e colonna d'acqua) nel Mar di Sardegna (Luglio 2013).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|--------------------------|-----------------------------|---------------|-----------------------------|
| Retinata plancton | Superficiale | MPM55 | N41 01.601 E7 54.868 |
| Retinata plancton | Superficiale | MPM56 | N40 59.656 E8 04.200 |
| Retinata plancton | Superficiale | MPM57 | N41 08.763 E8 04.378 |
| Retinata plancton | Superficiale | MPM58 | N41 09.890 E8 17.529 |
| Retinata plancton | Superficiale | MPM59 | N41 18.771 E8 04.902 |
| Retinata plancton | Superficiale | MPM60 | N41 14.464 E8 02.005 |
| Retinata plancton | Superficiale | MPM61 | N40 59.025 E8 13.259 |
| Retinata plancton | Superficiale | MPM62 | N41 19.381 E8 16.903 |
| Retinata plancton | Superficiale | MPM63 | N41 11.109 E8 30.101 |
| Retinata plancton | Superficiale | MPM64 | N41 22.187 E8 33.116 |
| Retinata plancton | Superficiale | MPM65 | N41 20.342 E8 43.425 |
| Retinata plancton | Superficiale | MPM66 | N40 49.707 E7 51.122 |
| Retinata plancton | Superficiale | MPM67 | N40 47.618 E8 01.834 |
| Retinata plancton | Superficiale | MPM68 | N40 59.683 E8 17.573 |
| Retinata plancton | Superficiale (Notturna) | MPM | N40 58.824 E8 10.793 |
| Retinata plancton | Verticale (Notturna) | MPP | N40 58.863 E8 10.162 |

Campionamenti di biopsie cutanee in cetacei free-ranging

Tab. 5. Tabella riassuntiva degli avvistamenti e campionamenti (biopsie cutanee) di cetacei in Mar di Sardegna (Luglio 2013).

| SPECIE | AVVISTAMENTI | DATA | n° BIOPSIE | COORDINATE |
|------------------------------|---------------|------------|------------|----------------------|
| <i>Stenella coeruleoalba</i> | 25 esemplari | 08/07/2013 | 2 | N41 03.445 E7 53.822 |
| <i>Balaenoptera physalus</i> | 3 esemplari | 09/07/2013 | 1 | N41 17.776 E8 01.945 |
| <i>Stenella coeruleoalba</i> | 10 esemplari | 09/07/2013 | 2 | N41 14.701 E8 11.398 |
| <i>Tursiops truncatus</i> | 10 esemplari | 09/07/2013 | - | N40 59.726 E8 11.539 |
| <i>Grampus griseus</i> | 2 esemplari | 09/07/2013 | - | N41 11.318 E8 15.696 |
| <i>Stenella coeruleoalba</i> | 100 esemplari | 10/07/2013 | 3 | N41 10.853 E8 12.432 |
| <i>Stenella coeruleoalba</i> | 150 esemplari | 10/07/2013 | 2 | N41 10.853 E8 12.432 |
| <i>Tursiops truncatus</i> | 5 esemplari | 10/07/2013 | - | N41 07.358 E8 19.987 |
| <i>Tursiops truncatus</i> | 15 esemplari | 11/07/2013 | - | N41 05.355 E8 21.553 |
| <i>Stenella coeruleoalba</i> | 200 esemplari | 12/07/2013 | 4 | N41 14.108 E8 11.950 |
| <i>Tursiops truncatus</i> | 5 esemplari | 12/07/2013 | 1 | N41 05.885 E8 21.257 |
| <i>Stenella coeruleoalba</i> | 200 esemplari | 13/07/2013 | 5 | N41 13.974 E8 29.369 |
| <i>Tursiops truncatus</i> | 15 esemplari | 13/07/2013 | - | N41 10.421 E8 28.092 |
| <i>Grampus griseus</i> | 3 esemplari | 13/07/2013 | 1 | N41 10.421 E8 28.092 |
| <i>Stenella coeruleoalba</i> | 150 esemplari | 14/07/2013 | 7 | N41 18.117 E8 25.136 |
| <i>Tursiops truncatus</i> | 3 esemplari | 14/07/2013 | 1 | N41 30.084 E8 51.660 |
| <i>Stenella coeruleoalba</i> | 100 esemplari | 15/07/2013 | 7 | N41 14.615 E8 01.706 |
| <i>Delphinus delphis</i> | 25 esemplari | 16/07/2013 | 2 | N40 49.582 E8 02.581 |
| <i>Delphinus delphis</i> | 20 esemplari | 16/07/2013 | 2 | N40 49.783 E8 02.334 |

Di seguito viene riportata la restituzione grafica mediante mappa dedicata degli avvistamenti di cetacei e relative biopsie effettuati nel Mar di Sardegna (Luglio 2013) (Fig.9)



Fig.9. Restituzione grafica mediante mappa degli avvistamenti di cetacei e relative biopsie effettuate nel Mar di Sardegna (Luglio 2013). Verde: tursiopo, rosa: balenottera comune, blu: stenella striata, rosso: grampo, viola: delfino comune, bianco: capodoglio.



Fig.10. Avvistamento di esemplari di delfino comune durante la compagnia di campionamento in Sardegna (16/07/2013).

3° Campagna - Mar Ligure

Le campagna svolta nel Mar Ligure è il risultato di tre periodi di attività di campionamento e monitoraggio svoltisi tra agosto e ottobre 2013. Lo scopo principale di questa campagna è stata quella di realizzare:

- **Campionamenti di plancton e microplastiche superficiali**
- **Campionamenti di plancton e microplastiche nella colonna d'acqua**
- **Campionamenti di biopsie cutanee in cetacei free-ranging**
- **Campionamenti di eufasiacei**
- **Monitoraggio delle macroplastiche**

Di seguito vengono riportati in dettaglio le tempistiche delle varie attività (agosto-ottobre 2013), l'area percorsa, le miglia percorse, il numero e la localizzazione dei campioni di plancton e microplastiche effettuati, il numero e la localizzazione degli avvistamenti di esemplari di cetacei ed il numero e la localizzazione delle biopsie effettuate.

1-3 Agosto 2013

Inizio attività: 01 Agosto 2013 (ore 12:00)

Fine attività: 03 Agosto 2013 (ore 16:00)

Miglia percorse: 120 miglia percorse di cui on effort 100,80

Campionamenti di plancton e microplastiche: 1 retinata superficiale

Avvistamenti esemplari di cetacei: n° 2 (gruppi) *Stenella coeruleoalba* e n° 15 esemplari di *Balaenoptera physalus*

Campionamenti effettuati: 2 biopsie *Stenella coeruleoalba* e 10 biopsie *Balaenoptera physalus*



Fig.11. Rotte effettuate durante la prima campagna svolta nel Mar Ligure (Agosto 2013) durante le quali è stato effettuato il survey e monitoraggio delle macroplastiche.

Campionamenti di plancton e microplastiche superficiali

Durante questo periodo, l'attività si è concentrata sul campionamento delle biopsie cutanee di balenottera comune ed è stato possibile effettuare solamente un campionamento di microplastiche e plancton. Per questo motivo un monitoraggio più ampio si è svolto nei successivi periodi dedicati a questa specifica attività.

Tab. 6 - Retinate per campionamento plancton e microplastiche in Mar Ligure (Agosto 2013).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|-------------------|--------------|--------|----------------------|
| Retinata plancton | Superficiale | MPM69 | N43 39.571 E7 48.825 |

Campionamenti di biopsie cutanee in cetacei free-ranging

Tab.7 - Avvistamenti e campionamento cetacei (biopsie cutanee) in Mar Ligure (Agosto 2013).

| SPECIE | AVVISTAMENTI | DATA | n° BIOPSIE | COORDINATE |
|------------------------------|--------------|------------|------------|----------------------|
| <i>Stenella coeruleoalba</i> | 30 | 01/08/2013 | 2 | N43 33.295 E7 53.429 |
| <i>Balaenoptera physalus</i> | 15 | 02/08/2013 | 10 | N43 23.236 E7 57.402 |
| <i>Stenella coeruleoalba</i> | 100 | 03/08/2013 | - | N43 30.448 E7 58.980 |

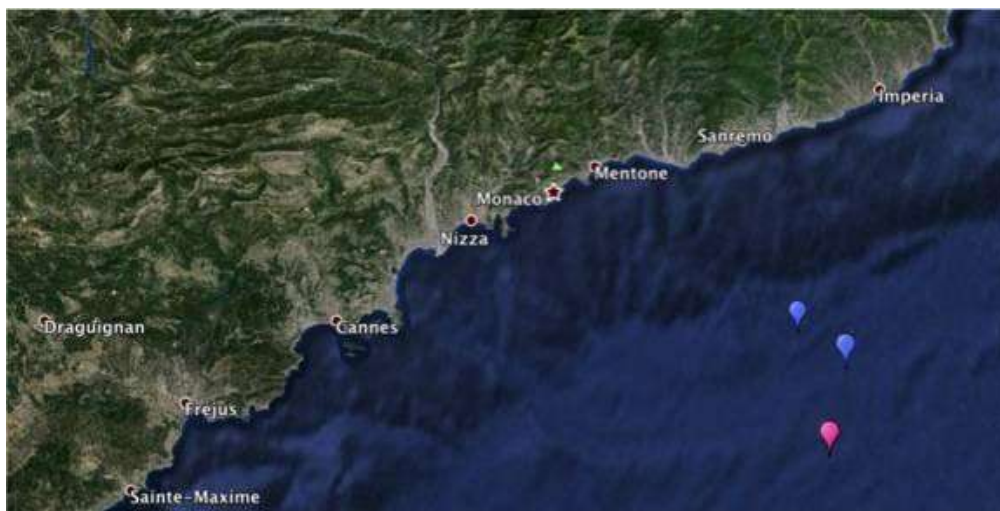


Fig.12. Restituzione grafica mediante mappa degli avvistamenti di cetacei e relative biopsie effettuate nel Mar Ligure (Agosto 2013). Rosa: balenottera comune, blu: stenella striata.

24-27 settembre 2013

Inizio attività: 25 Settembre 2013 (ore 9:00)

Fine attività: 27 Settembre 2013 (ore 17:00)

Miglia percorse: 196 miglia percorse di cui on effort 98,70

Campionamenti di plancton e microplastiche: 1 retinata superficiale

Avvistamenti esemplari di cetacei: n° 30 *Stenella coeruleoalba*, n° 1 *Balaenoptera physalus* e n°2 *Physeter macrocephalus*

I campionamenti di plancton e microplastiche superficiali sono stati condotti grazie all'appoggio della Marina Militare Italiana lungo una rotta che ha attraversato il Santuario Pelagos (Italia-

Monaco-Francia , vedi Fig. 13). Durante 3 giorni di navigazione, dal 24 al 27 di settembre, è stata seguita la rotta che unisce Livorno (Italia) con Tolone (Francia).



Fig.13. Rotta Livorno-Tolone.

Lungo tale rotta sono stati effettuati 7 campionamenti di plancton e microplastiche superficiali dei quali i dettagli vengono riportati (Tab. 8, Fig 14).

Tab. 8 - Retinate superficiali e nel Mar di Ligure (Settembre 2013).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|-------------------|--------------|--------|-----------------------|
| Retinata plancton | Superficiale | MPMM 1 | N43 30.144 E10 12.328 |
| Retinata plancton | Superficiale | MPMM 2 | N43 31.861 E09 49.445 |
| Retinata plancton | Superficiale | MPMM 3 | N43 28.376 E09 33.235 |
| Retinata plancton | Superficiale | MPMM 4 | N43 05.985 E07 40.117 |
| Retinata plancton | Superficiale | MPMM 5 | N43 02.376 E07 20.050 |
| Retinata plancton | Superficiale | MPMM 6 | N42 56.066 E06 52.408 |
| Retinata plancton | Superficiale | MPMM 7 | N43 02.993 E06 02.554 |



Fig.14. Mappa dei campionamenti di plancton e microplastiche superficiali. In rosso i punti dove sono state effettuate le retinate superficiali di giorno, in blu è evidenziato il punto del campionamento degli eufasiacei.

Durante i 3 giorni di navigazione sono stati avvistate tre specie di cetacei (Tab. 9, Fig. 15) ma, essendo questa attività strettamente effettuata per il monitoraggio delle microplastiche e plancton superficiale non sono stati eseguiti campionamenti degli esemplari *free-ranging*.

Tab. 9. Avvistamenti di cetacei effettuati nella seconda campagna in Mar Ligure (24 - 27 settembre 2013).

| SPECIE | AVVISTAMENTI | DATA | COORDINATE |
|-------------------------------|--------------|------------|-----------------------|
| <i>Physeter macrocephalus</i> | 2 | 26/09/2013 | N43 06.586 E07 42.998 |
| <i>Balaenoptera physalus</i> | 1 | 26/09/2013 | N43 02.795 E07 22.763 |
| <i>Stenella coeruleoalba</i> | 30 | 26/09/2013 | N43 02.020 E07 01.966 |

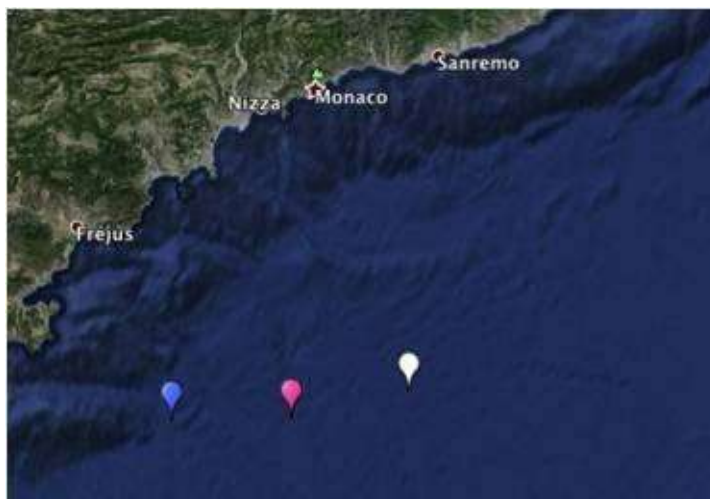


Fig.15. Restituzione grafica mediante mappa degli avvistamenti di cetacei nel Mar Ligure (Settembre 2013). Rosa: balenottera comune, blu: stenella striata., bianco: capodoglio.

7-9 ottobre 2013

Inizio attività: 7 Ottobre 2013 (ore 12:00)

Fine attività: 9 Ottobre 2013 (ore 12:00)

Miglia percorse: 113 miglia percorse di cui on effort 81,30

Campionamenti di plancton e microplastiche: 9 retinate superficiale, 5 retinate colonna d'acqua

Avvistamenti esemplari di cetacei: n° 15 *Stenella coeruleoalba*

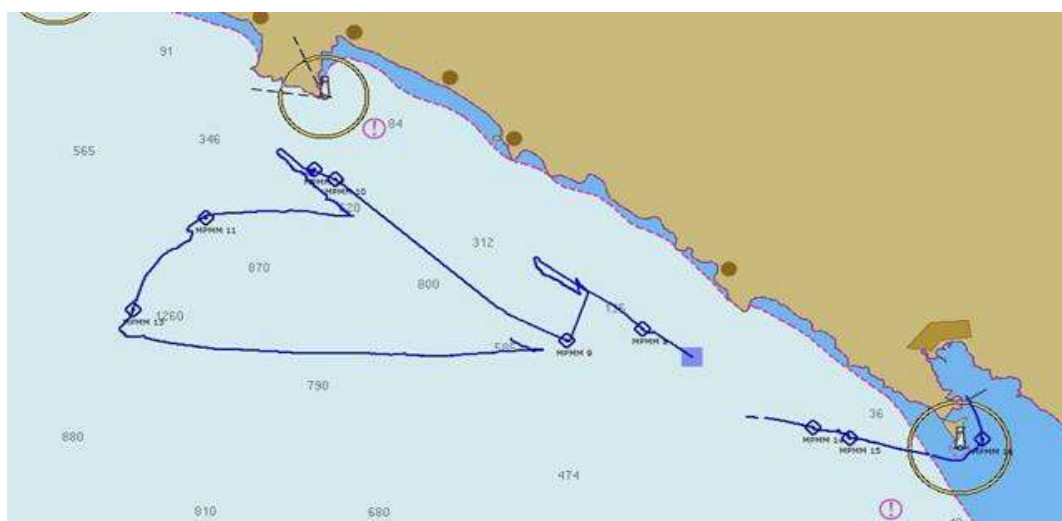


Fig.16. Rotta percorsa nel Mar Ligure (7-9 ottobre 2013).

Campionamenti di plancton e microplastiche superficiali e campionamenti lungo la colonna d'acqua (verticali) sono stati condotti grazie all'appoggio della Marina Militare Italiana lungo una rotta che ha attraversato il Santuario Pelagos nella zona del Mar Ligure prospiciente l'area delle Cinque Terre (vedi Fig. 16). Durante i 3 giorni di navigazione dal 7 al 9 ottobre è stata seguita la rotta che partendo dal porto di La Spezia costeggia la Riviera di Levante fino al promontorio di Portofino. Sono state individuate 9 stazioni di campionamento, 2 pelagiche e 7 costiere, riportate nella mappa della rotta percorsa (Tab. 10, Fig. 17).

Tab. 10. Retinate per campionamento plancton e microplastiche superficiali (9) e campionamenti nella colonna d'acqua (5) realizzate nella terza campagna in Mar Ligure (7-9 ottobre 2013).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|-------------------|--------------|---------|-----------------------|
| Retinata plancton | Superficiale | MPMM 8 | N44 07.076 E09 32.017 |
| Retinata plancton | Superficiale | MPMM 9 | N44 09.547 E09 26.359 |
| Retinata plancton | Superficiale | MPMM 10 | N44 14.039 E09 13.713 |
| Retinata plancton | Superficiale | MPMM 11 | N44 12.259 E09 06.028 |
| Retinata plancton | Superficiale | MPMM 12 | N44 14.441 E09 12.240 |
| Retinata plancton | Superficiale | MPMM 13 | N44 07.965 E09 01.643 |
| Retinata plancton | Superficiale | MPMM 14 | N44 02.484 E09 42.151 |
| Retinata plancton | Superficiale | MPMM 15 | N44 01.996 E09 44.386 |
| Retinata plancton | Superficiale | MPMM 16 | N44 01.940 E09 52.242 |
| Retinata plancton | Verticale | MPMV 1 | N44 07.076 E09 32.017 |
| Retinata plancton | Verticale | MPMV 2 | N44 14.039 E09 13.713 |
| Retinata plancton | Verticale | MPMV 3 | N44 12.259 E09 06.028 |
| Retinata plancton | Verticale | MPMV 4 | N44 07.965 E09 01.643 |
| Retinata plancton | Verticale | MPMV 5 | N44 02.484 E09 42.151 |

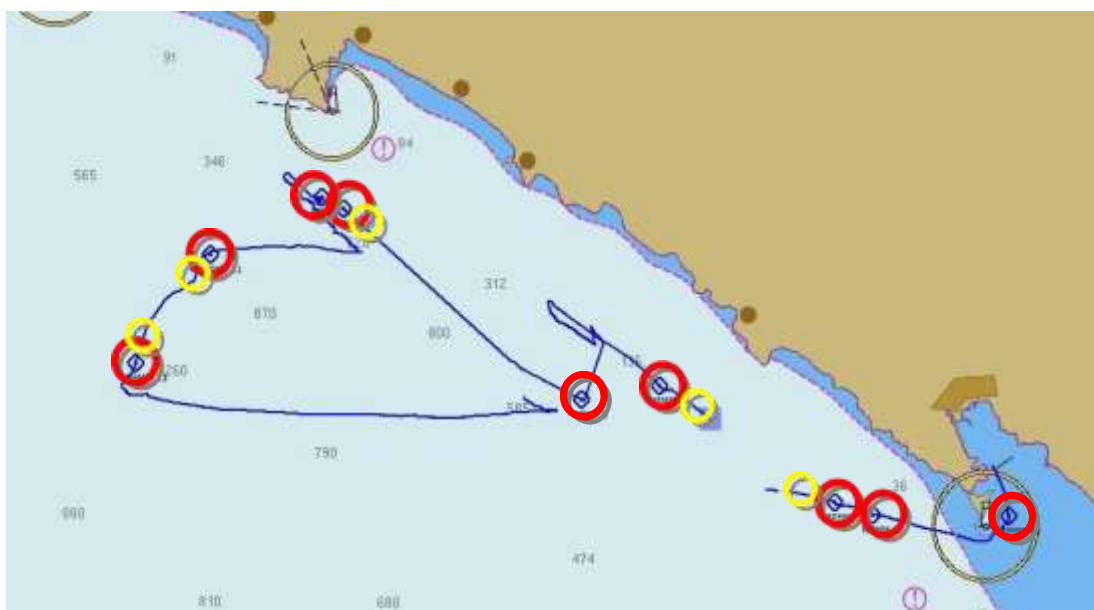


Fig.17. Mappa dei campionamenti di plancton e microplastiche superficiali. In rosso i punti dove sono state effettuate le retinate superficiali, in giallo le retinate nella colonna d'acqua (7-9 ottobre 2013)..

Vengono riportati di seguito gli avvistamenti di cetacei effettuati, grazie all' appoggio della Marina Militare Italiana, lungo una rotta che ha attraversato il Santuario Pelagos nella zona del Mar Ligure prospiciente alle Cinque Terre. Lungo tale rotta e' stato effettuato 1 avvistamento (stenella striata - gruppo) dei quali i dettagli vengono riportati in Tab.11 e Figura 18.

Tab. 11 – Avvistamenti di cetacei effettuati nella terza campagna in Mar Ligure (7 - 9 di ottobre).

| SPECIE | AVVISTAMENTI | DATA | COORDINATE |
|------------------------------|--------------|------------|----------------------|
| <i>Stenella coeruleoalba</i> | 15 | 08/10/2013 | N44 12.321 E9 18.992 |

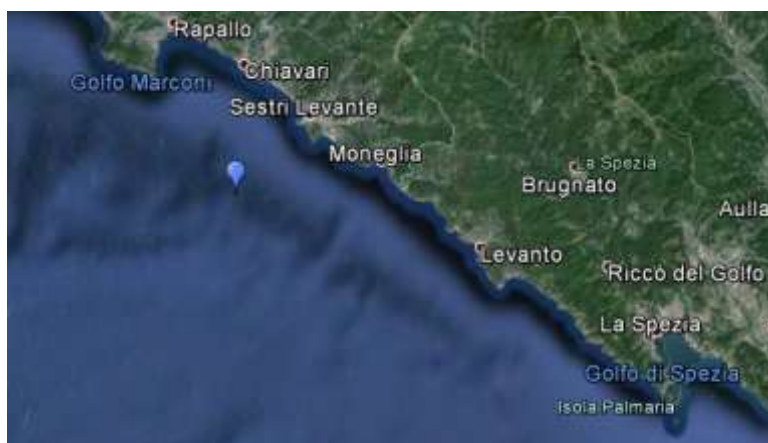


Fig.18. Restituzione grafica mediante mappa dedicata de gli avvistamenti di cetacei effettuati in Mar Ligure (7-9 ottobre 2013). Blu: stenella striata.

4° Campagna – Mar Tirreno (Arcipelago Toscano)

La quarta campagna (parte terminale del secondo anno del progetto), della durata di 1 giorno, si è svolta nell'area del Mar Tirreno (Arcipelago Toscano). Lo scopo principale di questa campagna è stato quello di realizzare la seguente tipologia di campionamento:

Campionamenti di plancton e microplastiche nella colonna d'acqua

Di seguito vengono riportati in dettaglio le tempistiche della campagna, l'area percorsa, le miglia percorse, il numero e la localizzazione dei campioni di plancton e microplastiche effettuati.

Inizio attività: 12 Novembre 2013 (ore 08:00)

Fine attività: 12 Novembre 2013 (ore 17:00)

Miglia percorse: 44 miglia percorse

Campionamenti di plancton e microplastiche: 4 retinate verticali

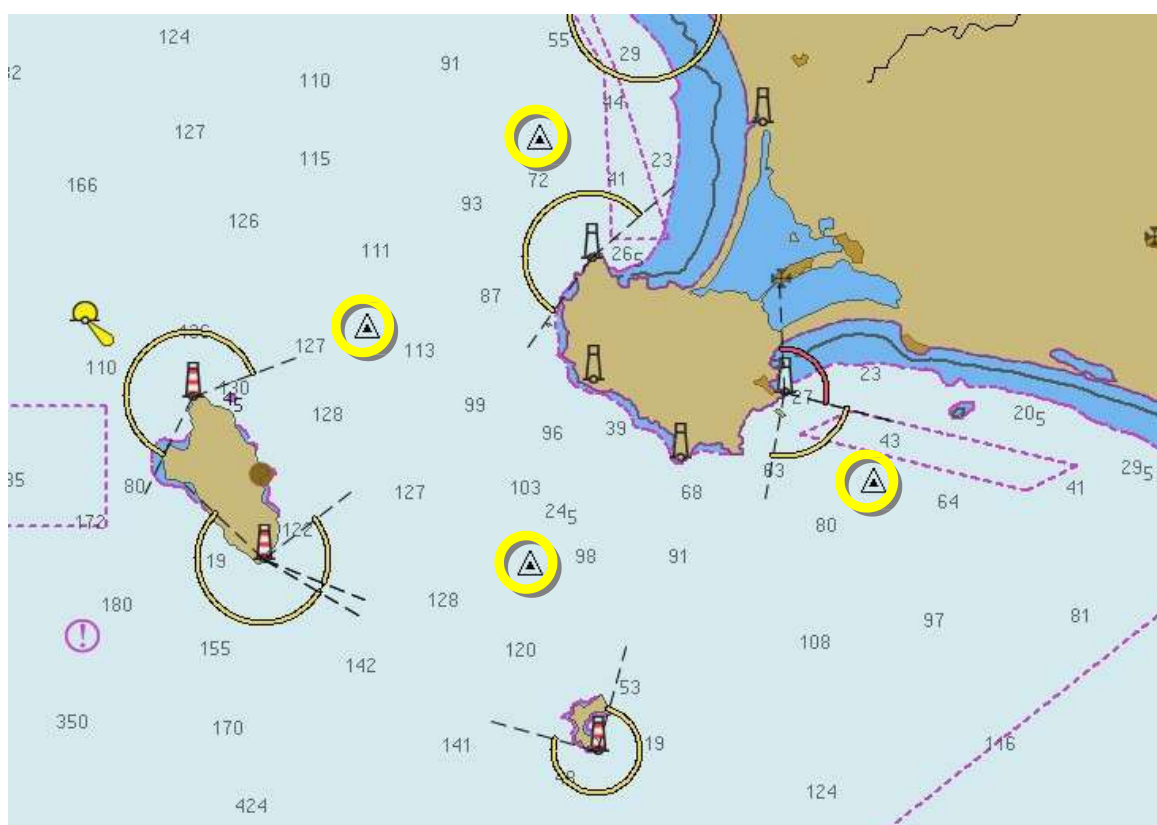


Fig.19. Mappa dei campionamenti di plancton e microplastiche nella colonna d'acqua (in giallo).

Tab. 12 - Retinate per campionamento plancton e microplastiche nella colonna d'acqua nel Mar Tirreno (Arcipelago Toscano)(Novembre 2013).

| CAMPIONAMENTO | TIPO | NUMERO | COORDINATE |
|-------------------|-----------|--------|-------------------------|
| Retinata plancton | Verticale | MPP70 | N 42 29.796 E 11 04.575 |
| Retinata plancton | Verticale | MPP71 | N 42 25.049 E 10 58.779 |
| Retinata plancton | Verticale | MPP72 | N 42 19.078 E 11 04.255 |
| Retinata plancton | Verticale | MPP73 | N 42 21.161 E 11 15.793 |

3. CAMPIONI DISPONIBILI PER INDAGINI ECOTOSSICOLOGICHE

Di seguito vengono riportati in dettaglio i campioni disponibili per le indagini eco tossicologiche.

Tab. 13. Campioni di plancton e microplastiche.

| Area | Data | Numero Retinate | Tipologia retinata | Eufasiacei |
|----------------------------------|---|-------------------------|--------------------------------|--|
| Mar Tirreno (Arcipelago Toscano) | Dicembre 2012 Novembre 2013 | 12 | 8 superficiali 4 verticali | - |
| Mar di Sardegna | Luglio 2013 | 14 diurne 2 notturne | 15 superficiali 1 verticale | 30 esemplari di <i>Meganyctiphanes norvegica</i> |
| Mar Ligure | Agosto 2013 Settembre 2013 Ottobre 2013 | 22 | 17 Superficiali 5 Verticali | 6 esemplari di <i>Meganyctiphanes norvegica</i> |



Fig.20. Immagini realizzate durante il campionamento effettuato con retino conico standard WP2.

Tab. 14. Biopsie cutanee di cetacei free-ranging campionate nel Santuario Pelagos.

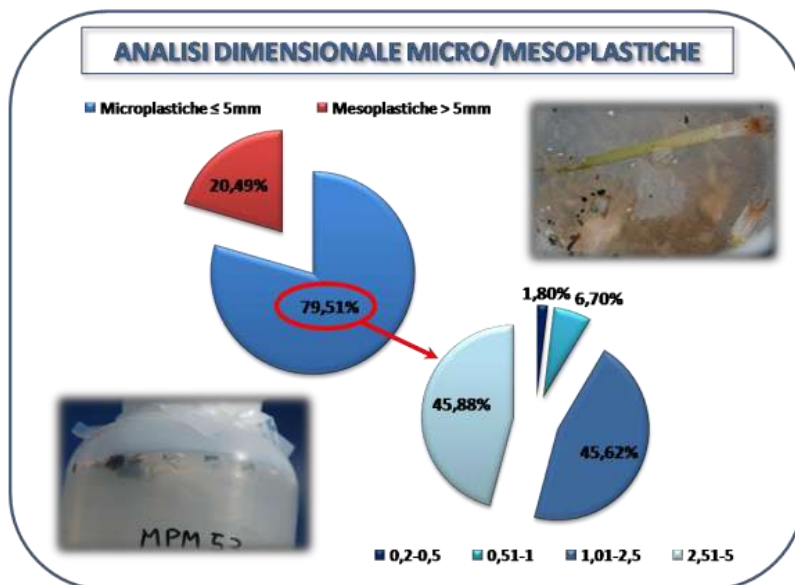
| Area | Specie | n° Biopsie | Codice UniSi |
|-------------------------------------|------------------------------|-------------------|---------------------|
| Mar Tirreno (Arcipelago Toscano) | <i>Balaenoptera physalus</i> | 1 | BPRT1 |
| Mar di Sardegna | <i>Stenella coeruleoalba</i> | 32 | AST7-38 |
| | <i>Balaenoptera physalus</i> | 1 | BPAS25 |
| | <i>Tursiops truncatus</i> | 2 | TTAS3-4 |
| | <i>Grampus griseus</i> | 1 | GGAS1 |
| | <i>Delphinus delphis</i> | 4 | DDAS1-4 |
| Mar Ligure | <i>Stenella coeruleoalba</i> | 2 | IST210-211 |
| | <i>Balaenoptera physalus</i> | 10 | BPL1-10 |



Fig.21. Esemplari di balenottera comune, grampo e stenella avvistati durante le campagne di campionamento.

3

RISULTATI E DISCUSSIONI



1. RISULTATI DELLE INDAGINI ECOTOSSICOLOGICHE E CONTEGGIO DELLE MICROPLASTICHE CAMPIONI DI PLANCTON/NEUSTON PRELEVATI IN DIVERSE AREE DEL SANTUARIO PELAGOS

Di seguito viene riportata l'analisi dei campioni di plancton e microplastiche relative sia al secondo anno di campionamento nelle tre sub-aree del Santuario Pelagos selezionate che integrando i risultati dei due anni di progetto (Tab.15-17).

a) Contenuto di microplastiche, zooplancton totale, MEHP e DEHP: dei 54 campioni superficiali di plancton/neuston analizzati, 44 contengono particelle di microplastiche (81%). Il valore più alto di particelle di microplastiche, rapportato ai m³ di acqua filtrata (3,33 items/m³), è stato riscontrato nel campione MPM53 (Arcipelago Toscano). La media totale degli items/m³ riscontrata nel totale delle retinate effettuate nelle tre aree del Santuario Pelagos è di 0,26 items/m³. Tra le diverse aree di campionamento risulta evidente come il valore medio del Mar Tirreno (Arcipelago Toscano) (0,80 items/m³) sia più alto rispetto alle altre due aree (Mar di Sardegna e Mar Ligure) nei diversi anni di campionamento. Per quanto riguarda i livelli di MEHP i campioni raccolti nel 2012 nel Mar di Sardegna sono quelli con il valore medio più elevato, mentre il valore medio più basso è risultato essere quello dei campioni provenienti dall'Arcipelago Toscano raccolti durante il 2012. Discorso diverso per il DEHP, per questo contaminante i valori medi più elevati e quelli più bassi sono stati riscontrati nella medesima area del Mar Ligure rispettivamente nel 2013 e nel 2012.

Durante i campionamenti e grazie a spiaggiamenti occasionali lungo le coste del Santuario Pelagos (Mar Ligure) sono stati rinvenuti esemplari di *Meganyctiphanes norvegica*, analizzati sia mediante tecniche di microscopia elettronica sia per valutare i livelli di ftalati (MEHP e DEHP). I livelli di MEHP e DEHP sono risultati di 9,56 e 15,33 ng/g peso secco, rispettivamente.

Tab.15. Valori delle microplastiche (items/m³), zooplancton totale (zoop/m³) e concentrazioni di MEHP e DEHP (ng/g) nelle retinate superficiali nelle diverse aree del Santuario Pelagos nell'anno 2012.

| Area di Campionamento | ID | items/m ³ | zoop/m ³ | MEHP ng/g | DEHP ng/g |
|---------------------------------|----------------|----------------------|---------------------|--------------|--------------|
| Mar di Sardegna 2012 | MPM27 | 0,44 | 150,99 | 60,57 | 0,00 |
| | MPM28 | 0,00 | 19,91 | 76,15 | 0,00 |
| | MPM29 | 0,07 | 109,50 | 96,27 | 0,00 |
| | MPM31 | 0,21 | 14,19 | 103,64 | 0,00 |
| | MPM32 | 0,04 | 129,18 | 50,80 | 0,00 |
| | MPM33 | 0,07 | 125,04 | 11205 | 0,00 |
| | MPM34 | 0,07 | 328,97 | 92,49 | 0,00 |
| | MPM35 | 0,11 | 50,14 | 234,93 | 87,28 |
| | MPM36 | 0,11 | 160,74 | 167,26 | 104,33 |
| | MPM37 | 0,00 | 143,05 | 94,60 | 0,00 |
| | MPM38 | 0,06 | 270,11 | 17,70 | 0,00 |
| | MPM39 | 0,00 | 185,39 | 24,43 | 0,00 |
| | MPM41 | 0,07 | 43,64 | 82,91 | 0,00 |
| MEDIA AREA | n=13 | 0,09 | 133,14 | 97,97 | 15,07 |
| Mar Ligure 2012 | MPM42 | 0,17 | 35,84 | 47,11 | 0,00 |
| | MPM43 | 0,07 | 21,77 | 82,96 | 0,00 |
| | MPM44 | 0,11 | 12,05 | 24,75 | 0,00 |
| | MPM45 | 0,83 | 14,27 | 39,27 | 37,22 |
| MEDIA AREA | n=4 | 0,30 | 20,98 | 48,52 | 9,30 |
| Arcipelago Toscano 2012 | MPM46 | 0,04 | 43,04 | 3,61 | 28,63 |
| | MPM47 | - | - | 0,00 | 10,68 |
| | MPM48 | 0,00 | 46,96 | 0,00 | 0,00 |
| | MPM49 | 0,00 | 29,32 | 2,32 | 0,00 |
| | MPM50 | - | - | 48,96 | 22,43 |
| | MPM51 | 0,44 | 440,46 | 59,28 | 14,89 |
| | MPM52 | 1,79 | 274,19 | 52,18 | 12,25 |
| | MPM53 | 3,33 | 236,02 | 10,03 | 10,74 |
| | MPM54 | 0,00 | 44,70 | - | - |
| MEDIA AREA | n=7/n=8 | 0,80 | 159,24 | 22,05 | 12,45 |

Tab.16. Valori delle microplastiche (items/m³) e zooplancton totale (zoop/m³) concentrazioni di MEHP e DEHP (ng/g) nelle retinate superficiali nelle diverse aree del Santuario Pelagos nell'anno 2013.

| Area di Campionamento | ID | items/m ³ | zoop/m ³ | MEHP ng/g | DEHP ng/g |
|-----------------------------|-------------|----------------------|---------------------|--------------|--------------|
| Mar di Sardegna 2013 | MPM55 | 0,14 | 990,17 | 18,25 | 16,50 |
| | MPM56 | 0,11 | 1172,75 | 18,15 | 12,30 |
| | MPM57 | 0,04 | 403,79 | 26,50 | 16,13 |
| | MPM58 | 0,07 | 8711,84 | 23,39 | 19,11 |
| | MPM59 | 0,25 | 1228,93 | 23,08 | 14,80 |
| | MPM60 | 1,69 | 259,83 | 31,90 | 14,49 |
| | MPM61 | 0,11 | 1488,76 | 22,52 | 12,70 |
| | MPM62 | 0,14 | 140,45 | 35,29 | 13,66 |
| | MPM63 | 0,00 | 242,28 | 29,26 | 26,05 |
| | MPM64 | 0,07 | 2212,08 | 33,08 | 18,23 |
| | MPM65 | 0,18 | 263,34 | 44,83 | 28,16 |
| | MPM66 | 0,39 | 474,02 | 35,77 | 27,90 |
| | MPM67 | 0,14 | 368,68 | 37,89 | 30,11 |
| | MPM68 | 0,00 | 5126,40 | 28,53 | 20,27 |
| MEDIA AREA | n=14 | 0,24 | 1648,81 | 29,18 | 19,31 |
| Mar Ligure 2013 | MPM69 | 0,00 | 4108,15 | 51,05 | 31,97 |
| | MPMM1 | 1,56 | 832,55 | 41,90 | 6,86 |
| | MPMM2 | 0,04 | 917,86 | 0,00 | 0,00 |
| | MPMM3 | 0,00 | 240,80 | 0,00 | 0,00 |
| | MPMM4 | 0,09 | 74,24 | 46,12 | 2,03 |
| | MPMM5 | 0,09 | 29,44 | 39,32 | 25,57 |
| | MPMM6 | 0,04 | 2,14 | 53,76 | 62,20 |
| | MPMM7 | 0,01 | 1978,27 | 48,39 | 221,27 |
| | MPMM8 | 0,13 | 13819,58 | 46,90 | 0,00 |
| | MPMM9 | 0,11 | 4886,41 | 47,79 | 61,17 |
| | MPMM10 | 0,03 | 727,96 | 49,09 | 62,70 |
| | MPMM11 | 0,01 | 1318,97 | 41,51 | 25,85 |
| | MPMM13 | 0,03 | 1675,23 | 48,32 | 63,62 |
| MPMM14 | 0,60 | 2226,35 | 29,63 | 70,92 | |
| MPMM15 | 0,07 | 2612,75 | 33,50 | 48,78 | |
| MPMM16 | 0,27 | 9549,71 | 18,93 | 34,73 | |
| MEDIA AREA | n=16 | 0,19 | 2812,52 | 37,77 | 43,12 |

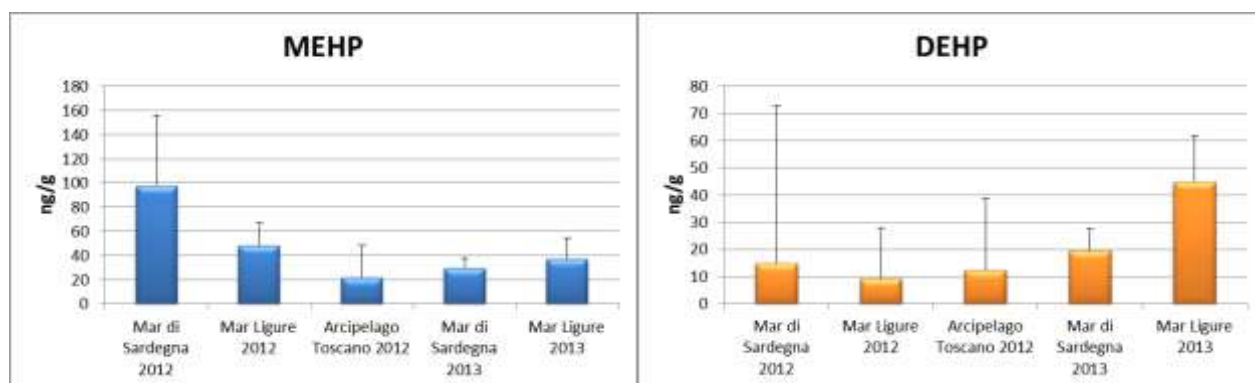


Fig.22. Livelli medi di MEHP e DEHP (ng/g peso secco) in campioni di plancton/neuston prelevati, suddivisi per area ed anno di campionamento.

Nelle retinate nella colonna d'acqua (Tab.17) non è stata rilevata la presenza di particelle di microplastiche. In questi campioni non è stato possibile valutare i livelli di MEHP e DEHP poiché la quantità di materia organica era insufficiente.

Tab.17. Valori delle microplastiche (items/m³) e zooplancton totale (zoop/m³) nelle retinate nella colonna d'acqua nelle diverse aree del Santuario Pelagos nell'anno 2012 e 2013.

| Area di Campionamento | ID | items/m ³ | zoop/m ³ |
|--------------------------------|------------|----------------------|---------------------|
| Mar di Sardegna 2012 | MPP30 | 0,00 | 27,38 |
| | MPP40 | 0,00 | 127,65 |
| MEDIA AREA | n=2 | 0,00 | 77,51 |
| Mar Ligure 2013 | MPMV1 | 0,00 | 1102,72 |
| | MPMV2 | 0,00 | 1266,05 |
| | MPMV3 | 0,00 | 864,88 |
| | MPMV4 | 0,00 | 811,31 |
| | MPMV5 | 0,00 | 566,24 |
| MEDIA AREA | N=5 | 0,00 | 922,24 |
| Arcipelago Toscano 2013 | MPP70 | 0,00 | 331,15 |
| | MPP71 | 0,00 | 1123,66 |
| | MPP72 | 0,00 | 1003,15 |
| | MPP73 | 0,00 | 175,47 |
| MEDIA AREA | N=4 | 0,00 | 658,36 |

b) Analisi dimensionale delle microplastiche: misurando le dimensioni di ogni singola plastica campionata durante le retinate superficiali è stato possibile suddividere gli items di plastica in due categorie: mesoplastiche (> 5mm) e microplastiche (≤ 5 mm). Le mesoplastiche non sono state considerate successivamente nell'analisi totale che ha fornito il dato di items/m³ (microplastiche) nelle diverse retinate. In Figura 23 sono riportati i valori % delle categorie di meso/microplastiche campionate nel totale delle retinate effettuate nel 2012/2013 e la suddivisione % delle microplastiche nelle 4 categorie dimensionali (0,2-0,5 ; 0,51-1 ; 1,01 - 2.5 ; 2,51 - 5 mm).

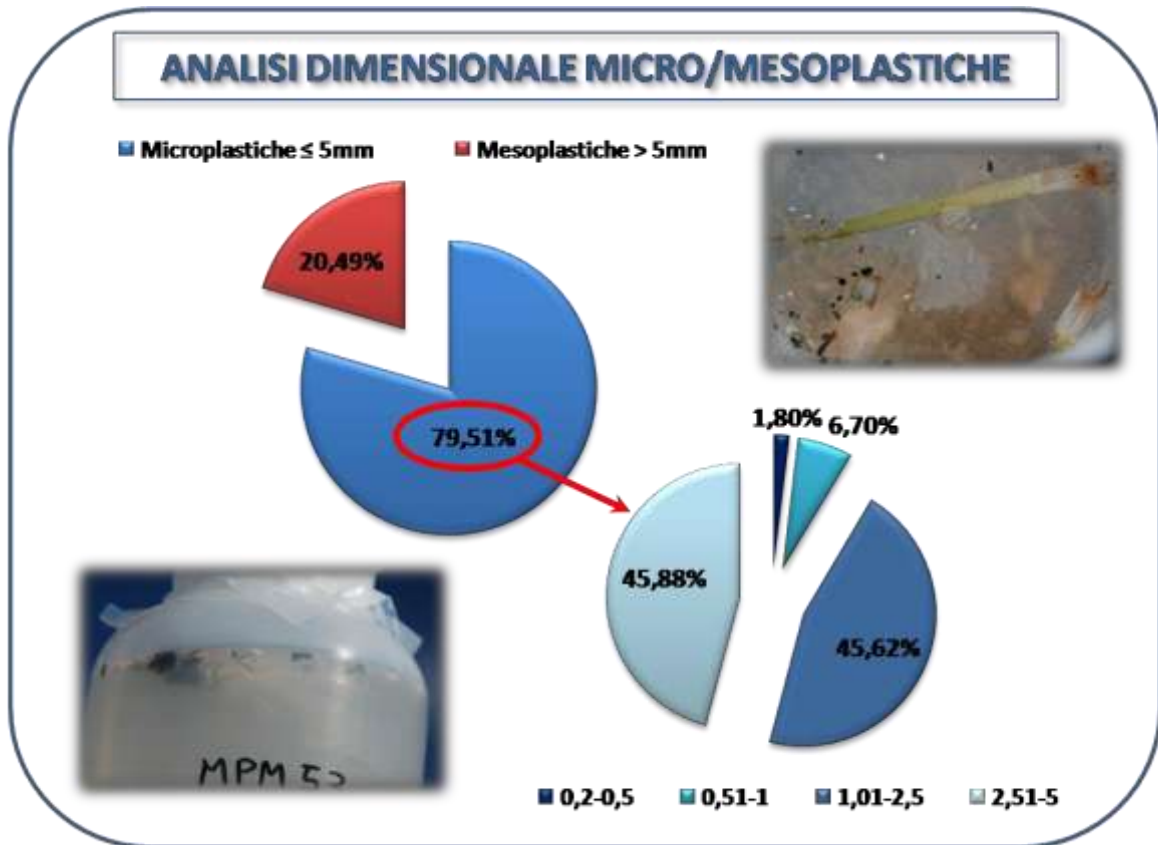


Fig.23. Valori % di microplastiche e mesoplastiche campionate nelle retinate superficiali 2012-2013.

Nella Fig.24 sono riportati i valori percentuale della presenza di micro e/o mesoplastiche suddivisi nelle aree e anni di campionamento. Tranne che per il campionamento nel Mar Ligure 2012 (60% di mesoplastiche) tutte le altre aree presentano valori più alti delle microplastiche rispetto alle mesoplastiche. Tale può essere influenzato dal numero basso di particelle di plastica ritrovato nelle 4 retinate effettuate.

Nella Fig.23 è stata inoltre effettuata una più accurata analisi dimensionale delle microplastiche campionate; le particelle di plastica sono state suddivise in 4 categorie in base alle loro dimensioni (0,2-0,5 ; 0,51-1 ; 1,01 - 2.5 ; 2,51 - 5 mm) per ottenere un'analisi più dettagliata delle dimensioni delle microplastiche. Possiamo notare come le categorie più presenti nelle 4 campagne di campionamento, escluso il Mar Ligure 2012, siano quelle comprensive degli items di dimensioni più grandi (1,01 - 2.5 mm; 2,51 - 5 mm) mentre la categoria delle microplastiche di dimensioni più piccole è presente solo in entrambe le campagne del 2013. Infine, come precedentemente descritto per micro/mesoplastiche, la suddivisione delle categorie in Mar Ligure 2012 è influenzata sicuramente dal numero basso di microplastiche campionate.

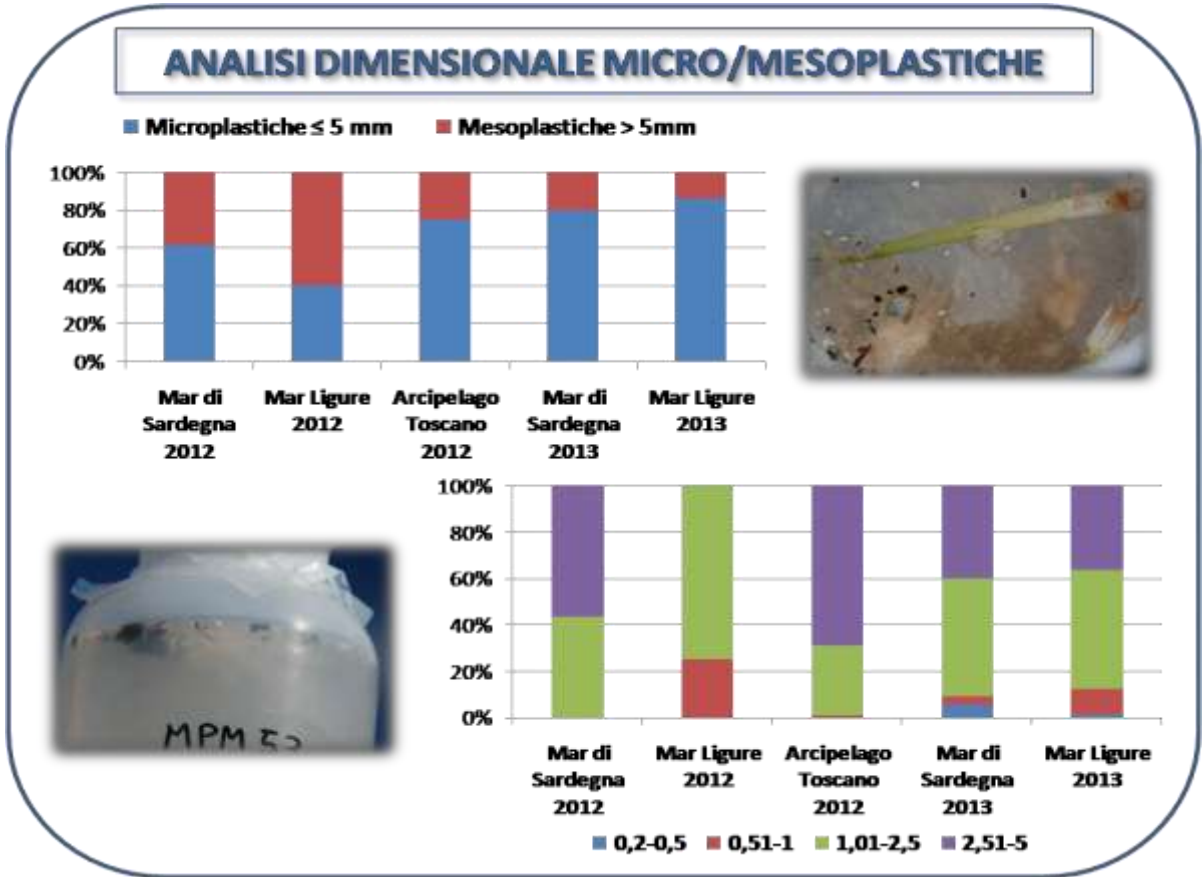


Fig. 24. Valori % delle categorie dimensionali di microplastiche suddivise nelle diverse aree ed anni di campionamento.

c) **Analisi delle principali componenti dello zooplancton:** nelle tabelle 18-24 vengono riportate le abbondanze (individui/m³) dei principali gruppi tassonomici identificati nelle retinate svolte nei due anni di progetto (2012-2013) nelle tre aree del Santuario Pelagos.

Tab.18. Organismi planctonici (individui/m³) campionati nel Mar Ligure nella campagna 2012.

| Mar Ligure 2012 | MPM42 | MPM43 | MPM44 | MPM45 |
|------------------------------|--------------|--------------|--------------|--------------|
| <i>Appendicolarie</i> | 2,16 | 0,25 | 0,34 | 0,17 |
| <i>Chetognati</i> | | 0,11 | | |
| <i>Cladoceri</i> | 0,00 | 12,50 | 2,64 | 0,50 |
| <i>Copepodi</i> | 17,75 | 8,46 | 5,34 | 13,44 |
| <i>Isopodi</i> | | | | 0,17 |
| <i>L. decapodi</i> | | 0,14 | | |
| <i>Molluschi pteropodi</i> | 13,61 | 0,11 | 0,34 | |
| <i>Sifonofori</i> | 0,33 | 0,21 | 0,29 | |
| <i>Uova Invertebrati</i> | 1,33 | | 3,10 | |
| <i>Uova Teleostei</i> | 0,66 | | | |
| Totale Zooplancton/m3 | 35,84 | 21,77 | 12,05 | 14,27 |

Tab.19. Organismi planctonici (individui/m³) campionati nel Mar Tirreno (Arcipelago Toscano) nella campagna 2012.

| Mar Tirreno 2012 | MPM46 | MPM48 | MPM49 | MPM51 | MPM52 | MPM53 |
|------------------------------|--------------|--------------|--------------|---------------|---------------|---------------|
| <i>Appendicolarie</i> | 4,77 | 7,45 | 5,60 | 105,46 | 47,95 | 46,95 |
| <i>Chetognati</i> | 2,98 | 1,06 | 0,40 | 6,20 | | 6,34 |
| <i>Cladoceri</i> | 5,57 | 4,38 | 3,20 | 13,96 | 38,96 | 16,50 |
| <i>Copepodi</i> | 27,63 | 28,86 | 15,33 | 286,92 | 161,82 | 105,96 |
| <i>Dolioli</i> | 0,60 | 0,59 | 0,53 | | 3,00 | 41,87 |
| <i>Foraminiferi</i> | | 1,06 | 0,67 | 1,55 | | |
| <i>Idromeduse</i> | 0,20 | 0,24 | | | | |
| <i>L. decapodi</i> | 0,10 | 0,24 | 0,13 | 1,55 | | |
| <i>L. pesci</i> | | 0,12 | 0,13 | | | |
| <i>Molluschi pteropodi</i> | 0,20 | 1,30 | 2,40 | 15,51 | 21,73 | 10,15 |
| <i>Radiolari</i> | 0,20 | | | | | 1,27 |
| <i>Salpe</i> | | 0,24 | 0,27 | | | |
| <i>Sifonofori</i> | 0,30 | 1,42 | 0,40 | 6,20 | 0,75 | 5,71 |
| <i>Uova Invertebrati</i> | | | | 3,10 | | |
| <i>Uova Teleostei</i> | 0,50 | | 0,27 | | | 1,27 |
| Totale Zooplancton/m3 | 43,04 | 46,96 | 29,32 | 440,46 | 274,19 | 236,02 |

Tab.20. Organismi planctonici (individui/m³) campionati nel Mar di Sardegna nella campagna 2012.

| Mar di Sardegna 2012 | MPM27 | MPM28 | MPM29 | MPP30 | MPM31 | MPM32 | MPM33 | MPM34 | MPM35 | MPM36 | MPM37 | MPM38 | MPM39 | MPP40 | MPM41 |
|------------------------------|---------------|--------------|---------------|--------------|--------------|---------------|---------------|---------------|--------------|---------------|---------------|---------------|---------------|---------------|--------------|
| <i>Appendicularie</i> | 25,59 | 1,12 | 20,89 | 3,38 | 0,32 | 61,10 | 34,76 | 28,37 | 6,39 | 64,64 | 39,33 | 61,22 | 54,07 | 2,21 | 2,00 |
| <i>Chetognati</i> | | | | 0,58 | 0,07 | | | | | | | | | | |
| <i>Cladoceri</i> | 17,72 | 2,98 | 18,31 | 0,29 | 0,70 | 3,79 | 17,91 | 13,76 | 11,24 | 10,81 | 0,74 | 16,41 | 14,75 | 4,43 | 1,58 |
| <i>Copepodi</i> | 17,84 | 3,65 | 40,43 | 19,04 | 10,74 | 52,46 | 54,95 | 233,99 | 19,24 | 68,82 | 94,87 | 180,49 | 103,72 | 108,47 | 27,81 |
| <i>Foraminiferi</i> | 0,33 | 0,11 | | | | 0,21 | | | | | | | | | |
| <i>Idromeduse</i> | 3,54 | 0,14 | 1,23 | 0,79 | | | | | | | | | | | |
| <i>L. decapodi</i> | | 0,56 | 1,11 | | | 1,47 | 10,53 | 3,37 | 1,40 | 5,65 | 1,47 | 8,20 | 2,21 | | |
| <i>L. echinodermi</i> | | 0,07 | | | | | | | | | | | | | |
| <i>L. pesci</i> | | | | | | 0,04 | 0,04 | 0,04 | | | | | 0,07 | | 0,04 |
| <i>L. policheti</i> | | 0,11 | | | | | | | | | | | | | |
| <i>Molluschi pteropodi</i> | 79,54 | 9,09 | 7,25 | 1,46 | 0,98 | 6,11 | 2,98 | 39,89 | 6,81 | 4,67 | 4,67 | 2,52 | 6,64 | 2,40 | 7,27 |
| <i>Policheti</i> | | | | 0,04 | | | 0,18 | | | | | | | | |
| <i>Salpe</i> | 0,11 | 0,21 | 1,35 | | | | | | | | | | | | |
| <i>Sifonofori</i> | 4,65 | 0,74 | 2,21 | 0,79 | 0,98 | | 2,11 | 1,12 | 1,12 | 2,46 | 0,49 | | 0,74 | 6,27 | 2,74 |
| <i>Uova Invertebrati</i> | | 0,98 | 1,47 | 0,33 | 0,32 | 2,74 | | 1,12 | 1,12 | 0,49 | 0,25 | 0,63 | 0,98 | | |
| <i>Uova Teleostei</i> | 1,66 | 0,14 | 15,24 | 0,67 | 0,07 | 1,26 | 1,58 | 7,30 | 2,81 | 3,20 | 1,23 | 0,63 | 2,21 | 0,37 | 2,21 |
| Totale Zooplancton/m3 | 150,99 | 19,91 | 109,50 | 27,38 | 14,19 | 129,18 | 125,04 | 328,97 | 50,14 | 160,74 | 143,05 | 270,11 | 185,39 | 124,15 | 43,64 |

Tab.21. Organismi planctonici (individui/m³) campionati nel Mar di Sardegna nella campagna 2013.

| Mar di Sardegna 2013 | MPM 55 | MPM 56 | MPM 57 | MPM 58 | MPM 59 | MPM 60 | MPM 61 | MPM 62 | MPM 63 | MPM 64 | MPM 65 | MPM 66 | MPM 67 | MPM 68 |
|------------------------------|---------------|----------------|---------------|----------------|----------------|---------------|----------------|---------------|---------------|----------------|---------------|---------------|---------------|----------------|
| <i>Anfipodi</i> | 3,51 | | | | | | | | | | | | | |
| <i>Appendicolarie</i> | | | 35,11 | 193,21 | | | 182,58 | 10,53 | | | 17,56 | | 17,56 | 2808,99 |
| <i>Chetognati</i> | 3,51 | 42,13 | 17,56 | | | | 28,09 | | | | | | | |
| <i>Cladoceri</i> | 488,06 | 610,96 | 87,78 | 509,36 | 895,37 | 91,29 | 1053,37 | 28,09 | 21,07 | 210,67 | | 245,79 | 87,78 | 280,90 |
| <i>Copepodi</i> | 284,41 | 428,37 | 193,12 | 87,82 | 52,67 | 105,34 | 42,13 | 24,58 | 7,02 | | 17,56 | 158,01 | 17,56 | 632,02 |
| <i>Efire</i> | | 28,09 | | | | | 14,04 | | | | | | 210,67 | |
| <i>Idromeduse</i> | | | | | | | 14,04 | | | | 17,56 | | | |
| <i>L.bivalvi</i> | 7,02 | | | | 17,56 | | | | | | | | | |
| <i>L.cirripedi</i> | | | | | | | | | | | | | | 70,22 |
| <i>L. decapodi</i> | | 7,02 | | | | 17,56 | 14,04 | | | 17,56 | | | | 1123,60 |
| <i>L. echinodermi</i> | | | | | | | | | 7,02 | | 17,56 | | | |
| <i>L. eufausiacei</i> | | | | 17,56 | | | | | | | | | | |
| <i>L.gasteropodi</i> | 17,56 | 49,16 | 35,11 | | 87,78 | 0,00 | 14,04 | | | | | | | 70,22 |
| <i>L. policheti</i> | | | | 87,82 | | | | 10,53 | 7,02 | | | | | |
| <i>Molluschi gasteropodi</i> | | | | 7517,48 | 175,56 | 28,09 | 126,40 | 66,71 | 189,61 | 1966,29 | 193,12 | 35,11 | 35,11 | 70,22 |
| <i>Nauplii copepodi</i> | | | | | | 17,56 | | | | | | | | |
| <i>Salpe</i> | | | | 228,33 | | | | | 7,02 | 17,56 | | | | |
| <i>Sifonofori</i> | 182,58 | 7,02 | 35,11 | 70,26 | | | | | 3,51 | | | 35,11 | | |
| <i>Uova Invertebrati</i> | 3,51 | | | | | | | | | | | | | |
| <i>Uova Teleostei</i> | | | | | | | | | | | | | | 70,22 |
| Totale Zooplancton/m3 | 990,17 | 1172,75 | 403,79 | 8711,84 | 1228,93 | 259,83 | 1488,76 | 140,45 | 242,28 | 2212,08 | 263,34 | 474,02 | 368,68 | 5126,40 |

Tab.22. Organismi planctonici (individui/m³) campionati nel Mar Ligure nella campagna 2013.

| Mar Ligure 2013 | MPM 69 | MPMM1 | MPMM2 | MPMM3 | MPMM4 | MPMM5 | MPMM6 | MPMM7 | MPMM8 | MPMM9 | MPMM10 | MPMM11 | MPMM13 | MPMM14 | MPMM15 | MPMM16 |
|------------------------------|----------------|---------------|---------------|---------------|--------------|--------------|--------------|----------------|-----------------|----------------|---------------|----------------|----------------|----------------|----------------|----------------|
| <i>Appendicularie</i> | 175,56 | 156,10 | 97,26 | | | | | 342,06 | 4094,69 | 401,62 | 18,20 | 95,26 | 52,35 | 68,15 | 270,28 | 181,32 |
| <i>Chetognati</i> | | 17,34 | | | 14,85 | | 0,53 | | 341,22 | 33,47 | 27,30 | 21,98 | 20,94 | | 108,11 | 241,76 |
| <i>Cladoceri</i> | 2808,99 | 381,59 | 601,77 | 63,37 | | | 0,27 | 986,29 | 2047,34 | 133,87 | 9,10 | 762,07 | 649,15 | 976,87 | 504,53 | 1994,56 |
| <i>Copepodi</i> | 842,70 | 190,79 | 206,67 | 158,42 | 59,39 | 22,08 | 1,34 | 621,42 | 7080,40 | 3781,95 | 473,17 | 366,38 | 910,90 | 976,87 | 1585,67 | 7011,18 |
| <i>Eufasiacei</i> | | 34,69 | | | | | | | | | | | | | | |
| <i>Foraminiferi</i> | | | 6,08 | 6,34 | | | | | | | | | 10,47 | | | |
| <i>Idromeduse</i> | 35,11 | | | | | | | | | | | | 10,47 | | | |
| <i>L.cirripedi</i> | | | | | | | | | | | | | | 22,72 | | |
| <i>L. decapodi</i> | | | | | | | | | | | | 7,33 | | | | |
| <i>L. echinodermi</i> | | | | | | | | | | | | | | 22,72 | 36,04 | |
| <i>L. eufausiacei</i> | | | | | | | | | | | 9,10 | | | | | |
| <i>L. pesci</i> | | | | | | | | | | 33,47 | | | | | | 60,44 |
| <i>L.gasteropodi</i> | | | 6,08 | 6,34 | | | | | | 368,15 | 9,10 | 21,98 | 10,47 | | 36,04 | |
| <i>L. policheti</i> | | | | | | | | | 85,31 | | | | | | 36,04 | |
| <i>L. pteropodi</i> | | 17,34 | | | | | | 5,70 | 85,31 | | 163,79 | 43,97 | | 22,72 | 36,04 | 60,44 |
| <i>Molluschi pteropodi</i> | | | | | | | | | | | | | | | | |
| <i>Molluschi gasteropodi</i> | 175,56 | | | | | | | | | | | | | | | |
| <i>Nauplii copepodi</i> | 70,22 | | | 6,34 | | 3,68 | | | 85,31 | 33,47 | | | 10,47 | 45,44 | | |
| <i>Ostracodi</i> | | | | | | 3,68 | | | | | | | | 45,44 | | |
| <i>Radiolari</i> | | | | | | | | | | 33,47 | | | | | | |
| <i>Salpe</i> | | | | | | | | | | | 9,10 | | | 22,72 | | |
| <i>Sifonofori</i> | | | | | | | | 5,70 | | 33,47 | | | | 22,72 | | |
| <i>Uova Teleostei</i> | | 34,69 | | | | | | 17,10 | | 33,47 | 9,10 | | | | | |
| Totale Zooplancton/m3 | 4108,15 | 832,55 | 917,86 | 240,80 | 74,24 | 29,44 | 2,14 | 1978,27 | 13819,58 | 4886,41 | 727,96 | 1318,97 | 1675,23 | 2226,35 | 2612,75 | 9549,71 |

Tab.23. Organismi planctonici (individui/m³) campionati nel Mar Ligure nella campagna 2013.

| Mar Ligure 2013 | MPMV1 | MPMV2 | MPMV3 | MPMV4 | MPMV5 |
|------------------------------|-----------------|-----------------|----------------|----------------|----------------|
| <i>Appendicularie</i> | 180 ,90 | 56 ,50 | 168 ,89 | 143 ,21 | 86 ,72 |
| <i>Calyptopsis</i> | | 5 ,14 | | 20 ,20 | 15 ,07 |
| <i>Cladoceri</i> | 45 ,00 | 33 ,38 | 68 ,67 | 87 ,08 | 53 ,69 |
| <i>Copepodi</i> | 739 ,97 | 1109 ,40 | 536 ,38 | 457 ,47 | 348 ,08 |
| <i>Doliolidi</i> | | | 1 ,86 | | |
| <i>Foraminiferi</i> | 13 ,11 | | 3 ,71 | | |
| <i>Idromeduse</i> | 9 ,20 | 7 ,70 | 3 ,71 | 13 ,86 | 8 ,16 |
| <i>L. decapodi</i> | 35 ,18 | | 12 ,99 | 0 ,00 | |
| <i>L. pteropodi</i> | 42 ,17 | 23 ,11 | 22 ,27 | 47 ,92 | 34 ,81 |
| <i>Policheti</i> | 1 ,26 | 2 ,57 | | 3 ,57 | |
| <i>Salpe</i> | | 17 ,98 | | | |
| <i>Sifonofori</i> | 27 ,95 | 5 ,14 | 37 ,12 | 28 ,24 | 15 ,11 |
| <i>Uova Teleostei</i> | 7 ,99 | 5 ,14 | 9 ,28 | 9 ,75 | 4 ,61 |
| Totale Zooplancton/m3 | 1102 ,72 | 1266 ,05 | 864 ,88 | 811 ,31 | 566 ,24 |

Tab.24. Organismi planctonici (individui/m³) campionati nel Mar Tirreno(Arcipelago Toscano) nella campagna 2013.

| Mar Tirreno 2013 | MPP70 | MPP71 | MPP72 | MPP73 |
|------------------------------|----------------|-----------------|-----------------|----------------|
| <i>Appendicularie</i> | 180 ,90 | 56 ,50 | 168 ,89 | 143 ,21 |
| <i>Appendicularie</i> | 14 ,51 | 78 ,85 | 61 ,46 | 9 ,65 |
| <i>Chetognati</i> | 4 ,28 | 69 ,89 | 54 ,60 | 0 ,57 |
| <i>Copepodi</i> | 184 ,38 | 651 ,14 | 670 ,27 | 154 ,46 |
| <i>Dolioli</i> | 1 ,25 | 14 ,93 | 31 ,79 | |
| <i>Foraminiferi</i> | 6 ,94 | | 0 ,09 | |
| <i>Idromeduse</i> | 3 ,47 | | | |
| <i>L. decapodi</i> | | 2 ,99 | 0 ,47 | 1 ,42 |
| <i>L. echinodermi</i> | | 0 ,60 | | |
| <i>L. eufausiacei</i> | | | 0 ,09 | |
| <i>L. pesci</i> | | 0 ,60 | | |
| <i>L. policheti</i> | | 2 ,39 | 0 ,09 | 0 ,57 |
| <i>L. pteropodi</i> | 6 ,94 | 267 ,03 | 173 ,95 | 4 ,83 |
| <i>Ostracodi</i> | 8 ,68 | 1 ,79 | 0 ,19 | |
| <i>Salpe</i> | | 5 ,38 | | |
| <i>Sifonofori</i> | 86 ,81 | 21 ,51 | 9 ,96 | 3 ,98 |
| <i>Uova Teleostei</i> | 13 ,89 | 6 ,57 | 0 ,19 | 0 ,00 |
| Totale Zooplancton/m3 | 331 ,15 | 1123 ,66 | 1003 ,15 | 175 ,47 |

d) Analisi attraverso *Geographic Information System (GIS)*: i risultati ottenuti dal campionamento di plancton e microplastiche nell'area del Mar di Sardegna e del Mar Ligure durante le campagne di campionamento del 2011, 2012 e 2013 sono stati elaborati con il metodo di interpolazione della media pesata sull'inverso della distanza (IDW) tramite il sistema di informazione geografica ArcGIS. Questo ha permesso di realizzare delle mappe di distribuzione dei frammenti di microplastiche (items/m^3) e delle specie di zooplancton ($\text{ind zoopl}/\text{m}^3$). Questo ha permesso di evidenziare le aree con minore e maggiore presenza delle variabili considerate (Fig. 25)

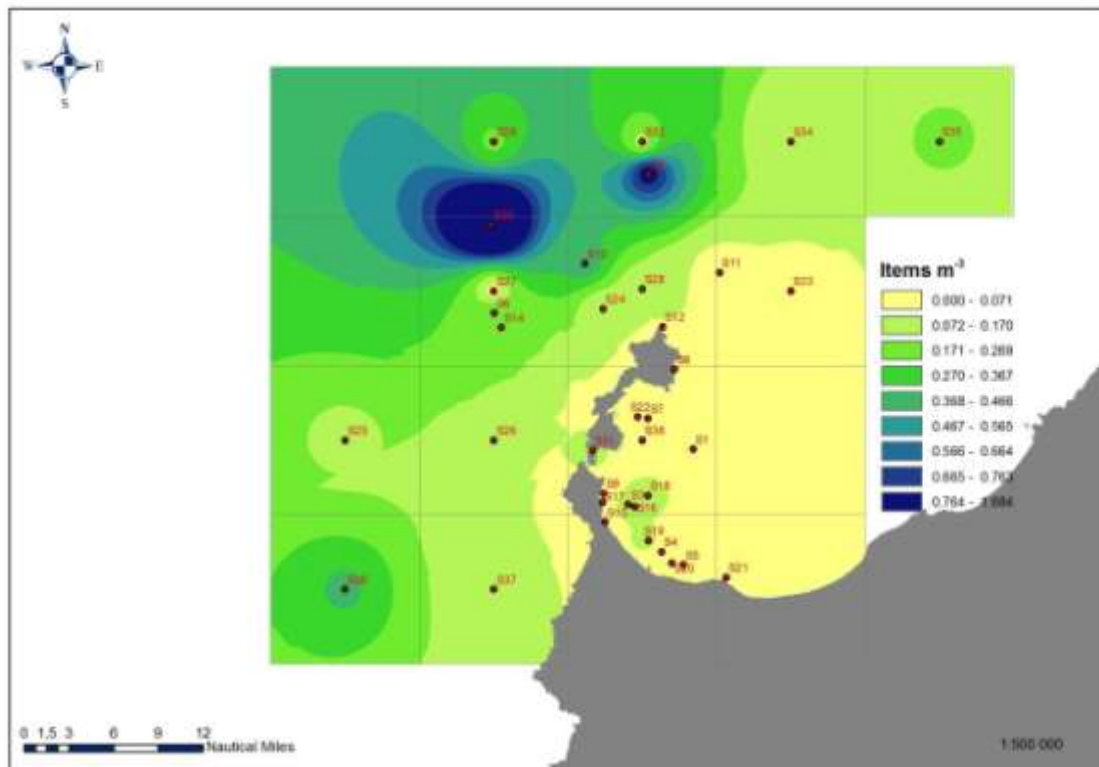


Fig. 25.Elaborazione grafica tramite GIS dell'abbondanza delle microplastiche (items/m^3) nel Mar di Sardegna (2011, 2012, 2013). In rosso i punti di campionamento.

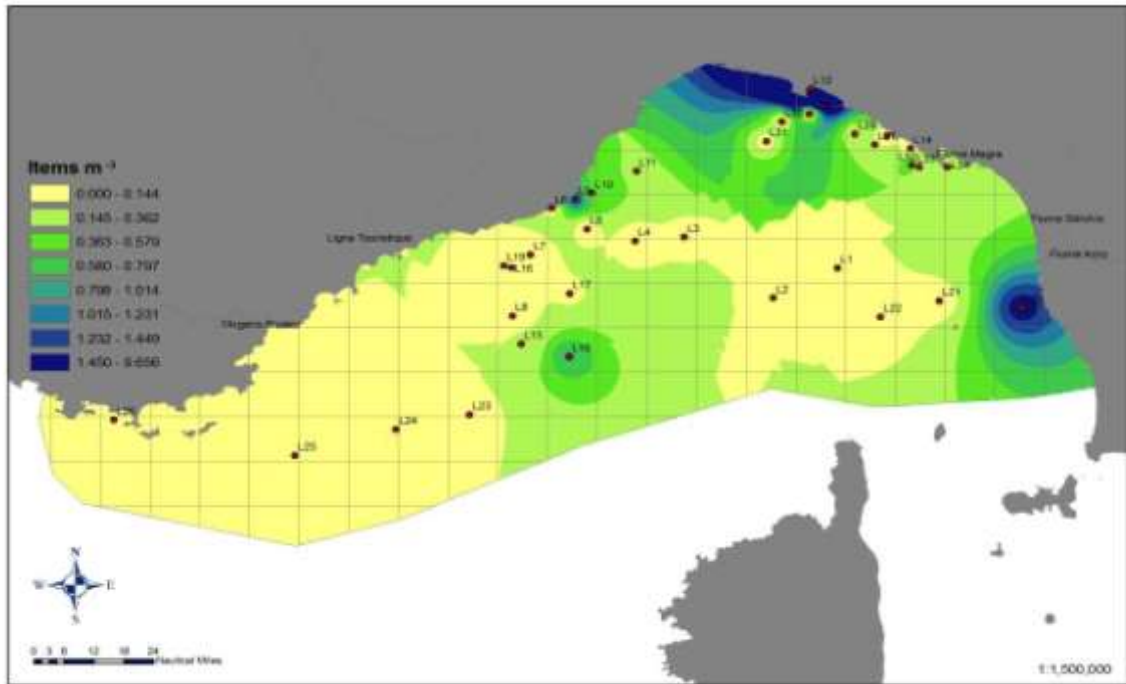


Fig. 26. Elaborazione grafica tramite GIS dell'abbondanza delle microplastiche (items/m³) nel Mar Ligure (2011, 2012, 2013). In rosso i punti di campionamento.

Inoltre, è stato possibile realizzare delle mappe di distribuzione e abbondanza per le specie zooplanctoniche individuate nei campioni (Appendicolarie, Cladoceri, Copepodi, Foraminiferi, Larve di decapodi, Larve di pteropodi, Policheti, Salpe, Sifonofori, Uova invertebrati, Uova teleostei) in modo da visualizzare in modo diretto le differenze e le similitudini della distribuzione tra le singole specie. Le mappe della distribuzione dello zooplancton specie per specie sono riportate nell'annesso 1.

2. RISULTATI DEGLI AVVISTAMENTI DELLE MACROPLASTICHE E DELLA MESSA A PUNTO DEL PROTOCOLLO “SURVEY MONITORAGGIO MARINE LITTER IN MARE”

Come già descritto nella sezione metodologica, la tecnica più utilizzata per la quantificazione ed il monitoraggio dei rifiuti marini galleggianti è l'osservazione visiva dalle imbarcazioni.

Nell'ambito del presente progetto è stato effettuato un monitoraggio dei rifiuti galleggianti nelle aree del Santuario Pelagos sfruttando sia le imbarcazioni utilizzate per la raccolta delle biopsie cutanee di cetacei e campioni di plancton e microplastiche, sia navi di opportunità che seguivano transetti predefiniti. L'osservazione è stata effettuata come descritta precedentemente prendendo in considerazione i rifiuti galleggianti osservati entro i 20 metri dall'imbarcazione, poiché ad una distanza maggiore gli oggetti sono difficilmente classificabile. La scheda utilizzata per la raccolta dei dati (riportata nella sezione metodologica) è stata ripresa e modificata dal protocollo NOAA (NOAA Form 57-11-14 (6-12)). Poiché lo scopo del progetto prevedeva solo il conteggio di macroplastiche, è stata effettuata successivamente un'elaborazione per il calcolo dei rifiuti galleggianti, tenendo in considerazione solo gli oggetti definiti come “plastiche” nella scheda. I risultati vengono riportati di seguito nelle tabelle 25 e 26.

Tab. 25. Risultati del survey delle macroplastiche nel Mar di Sardegna 2013 rapportate ai km² monitorati.

| Mar di Sardegna 2013 | | | | | |
|-----------------------------|---------------------------|-----------------------------|-----------------------|-----------------------|-----------------------------|
| Data | Miglia (On-Effort) | Items Macroplastiche | km (On-Effort) | km² | Items/km² |
| 08/07/2013 | 39 | 42 | 72.23 | 2.89 | 14.54 |
| 09/07/2013 | 34 | 176 | 62.97 | 2.52 | 69.88 |
| 10/07/2013 | 24 | 127 | 44.45 | 1.78 | 71.43 |
| 11/07/2013 | 45 | 120 | 83.34 | 3.33 | 36.00 |
| 12/07/2013 | 50 | 310 | 92.60 | 3.70 | 83.69 |
| 13/07/2013 | 35 | 57 | 64.82 | 2.59 | 21.98 |
| 14/07/2013 | 33 | 80 | 61.12 | 2.44 | 32.72 |
| 15/07/2013 | 48 | 104 | 88.90 | 3.56 | 29.25 |
| 16/07/2013 | 33 | 79 | 61.12 | 2.44 | 32.32 |
| Totale | 341 | 1095 | 631.53 | 25.25 | - |
| Media | - | - | - | - | 43.53 |

Tab.26. Risultati del survey delle macroplastiche nel Mar di Ligure 2013 rapportate ai km² monitorati.

| Mar Ligure 2013 | | | | | |
|------------------------|---------------------------|-----------------------------|-----------------------|-----------------------|-----------------------------|
| Data | Miglia (On-Effort) | Items Macroplastiche | km (On-Effort) | km² | Items/km² |
| 01/08/2013 | 40 | 241 | 74.08 | 2.96 | 81.33 |
| Data | Miglia (On-Effort) | Items Macroplastiche | km (On-Effort) | km² | Items/km² |
| 25/09/2013 | 9.10 | 46 | 16.85 | 0.34 | 136.47 |
| 26/09/2013 | 15.19 | 24 | 28.13 | 0.56 | 42.66 |
| Totale | 24.29 | 70.00 | 44.99 | 0.90 | - |
| Media | - | - | - | - | 77.80 |

3. RISULTATI DELLE DIAGNOSI ECOTOSSICOLOGICHE SUL POTENZIALE IMPATTO DELLE MICROPLASTICHE E DERIVATI TOSSICOLOGICAMENTE ATTIVI SU *B. PHYSALUS* NELLE TRE AREE DEL SANTUARIO PELAGOS.

Di seguito vengono riportati i risultati dei livelli di contaminanti e delle risposte dei biomarkers analizzati sulle 12 biopsie cutanee di balenottera comune campionate nel Santuario Pelagos (luglio-novembre 2013) per le diagnosi ecotossicologiche del potenziale impatto delle microplastiche su *B. physalus*.

I risultati ottenuti sono stati analizzati e discussi tenendo in considerazione il sesso degli individui (maschi *versus* femmine) per tutte le aree del Santuario Pelagos e l'anno di campionamento (2012 *versus* 2013) solo per il Mar Ligure. Non è stata presa in considerazione la variabile area perché nell'Arcipelago Toscano ed nel Mar di Sardegna gli esemplari campionati sono stati solo uno per area.

Vengono inoltre considerati singolarmente tutti i parametri analizzati: livelli di ftalati, organoclorurati, espressione proteica del CYP1A1 e CYP2B, perossidazione lipidica, espressione genica del PPAR A, PPAR G, AhR, CYP1A ed ER1. Inoltre, i risultati sono stati analizzati correlando i valori dei livelli di contaminanti con le risposte biologiche ed i diversi biomarker tra loro.

a) Analisi complessiva Maschi vs Femmine

-Analisi dei contaminanti

Livelli di ftalati

Nella porzione del blubber sottocutaneo delle biopsie cutanee di balenottera sono state valutate le concentrazioni dello ftalato mono-2-etilesilftalato (MEHP), principale metabolita del di-2-etilesilftalato (DEHP) composto additivo della plastica.

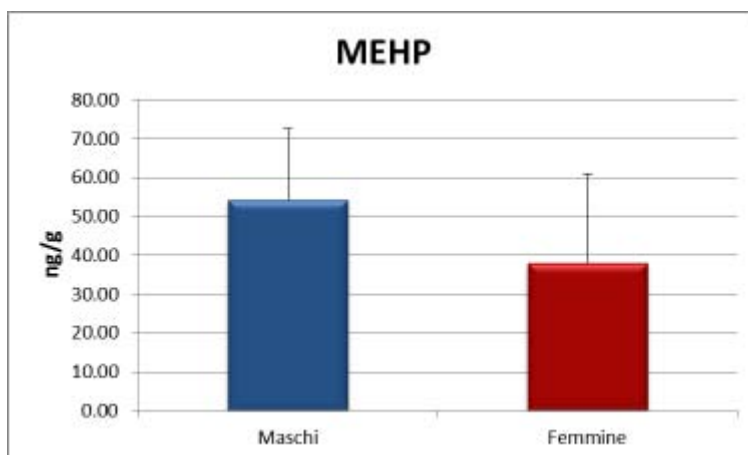


Fig.27. Livelli medi di MEHP (ng/g peso secco) in esemplari maschi (n=3) e femmine (n=4) di balenottera comune del Santuario Pelagos (2013).

I valori più elevati di MEHP sono stati riscontrati negli esemplari di sesso maschile, in cui il valore medio risulta essere 54,35 ng/g, mentre nelle femmine il valore medio riscontrato è 38,12 ng/g. Tale differenza non è tuttavia statisticamente significativa (Fig. 27).

Livelli degli organoclorurati

Nella porzione del blubber sottocutaneo delle biopsie cutanee di balenottera sono state valutate le concentrazioni dei composti organoclorurati DDT (e i suoi metaboliti) e PCB (30 congeneri), composti veicolati dalle particelle di plastica.

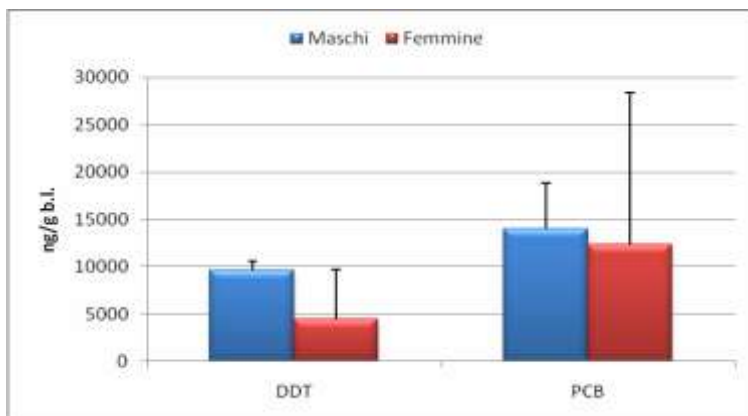


Fig.28. Livelli medi di organoclorurati (PCB totali – DDT e metaboliti) in esemplari maschi (n=6) e femmine (n=6) di balenottera comune del Santuario Pelagos (2013).

Per entrambe le categorie di OC indagate le concentrazioni più elevate dei contaminanti sono state evidenziate negli esemplari di sesso maschile sebbene tali differenze non siano statisticamente significative nella variabile sesso (Fig. 28).

- Risposte biologiche (biomarkers)

Nella porzione di derma delle biopsie cutanee sono state valutate le analisi delle risposte biomarker del citocromo P450 1A1/2B mediante western blot, la perossidazione lipidica e l'espressione genica del PPAR A, PPAR G, AhR, CYP1A e dell' ER1 mediante PCR Real-Time quantitativa.

- Livelli di CYP1A1 e CYP2B

Per entrambe le isoforme del citocromo P450 la maggiore induzione proteica è stata evidenziata negli esemplari di sesso maschile senza evidenziare però in entrambi i citocromi differenze statisticamente significative nella variabile sesso. Questo dato appare correlato ad i livelli di OC, che appaiono più alti nell'individui di sesso maschile (Fig. 29).

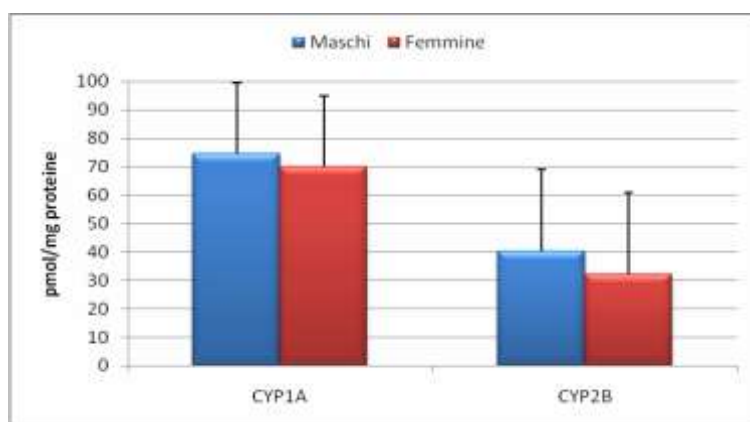


Fig.29. Livelli proteici di medi CYP1B e CYP2B in esemplari maschi (n=6) e femmine (n=5) di balenottera comune del Santuario Pelagos.

- Livelli di Perossidazione Lipidica

I dati riguardanti la perossidazione lipidica, biomarker di stress ossidativo, confermano le evidenze ottenute con l'analisi delle isoforme del citocromo; un valore moderatamente più elevato negli individui di sesso maschile rispetto alle femmine (Fig. 30).

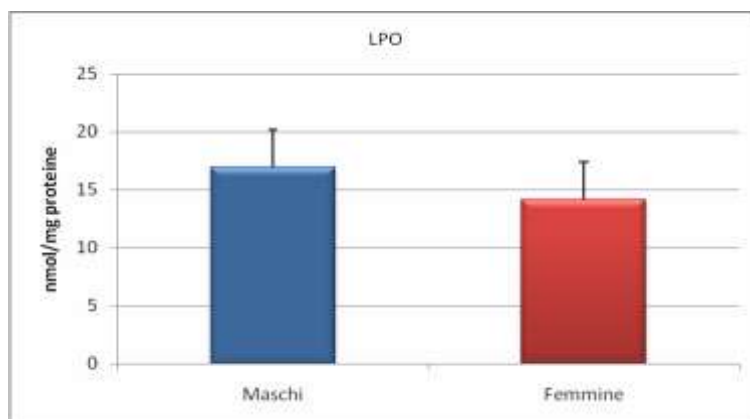


Fig.30. Livelli medi di perossidazione lipidica (LPO) in esemplari maschi (n=6) e femmine (n=6) di balenottera comune del Santuario Pelagos.

- Livelli di espressione genica

l'analisi di cinque geni biomarker mediante valutazione delle variazioni dell'espressione genica ha evidenziato come per ogni marcatore l'espressione più elevata risulti nelle femmine rispetto ai maschi. In particolare, una differenza statisticamente significativa ($p < 0.05$) risulta per le differenze di espressione dei geni CYP1A e ER1 tra i due sessi (Fig. 31).

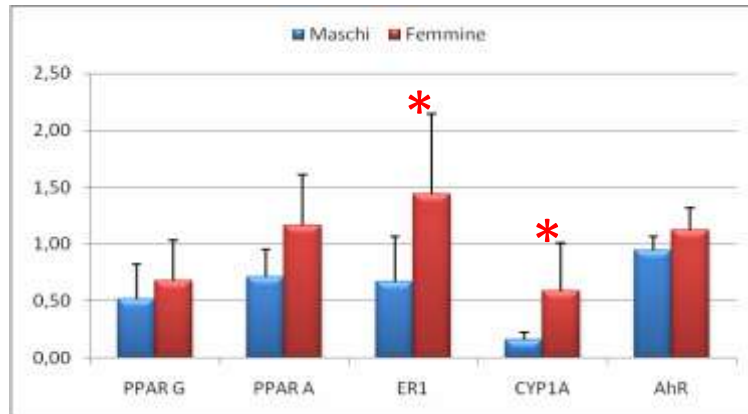


Fig.31. Livelli medi di espressione genica dei geni PPAR G, PPAR A, ER1, CYP1A e AhR in esemplari maschi (n=6) e femmine (n=6) di balenottera comune del Santuario Pelagos. * indica differenze statisticamente significative ($p < 0.05$) tra i sessi.

b) Analisi dei livelli di contaminanti e delle risposte dei biomarker per singolo individuo di *B. physalus*

- Analisi dei Contaminanti - Livelli degli organoclorurati

Per entrambe le categorie di OC indagate le concentrazioni più elevate dei contaminanti sono state evidenziate negli esemplari di sesso maschile ad eccezione dell'individuo BL10. In tutti i casi i valori dei PCB sono superiori a quelli dei DDT (Fig. 32).

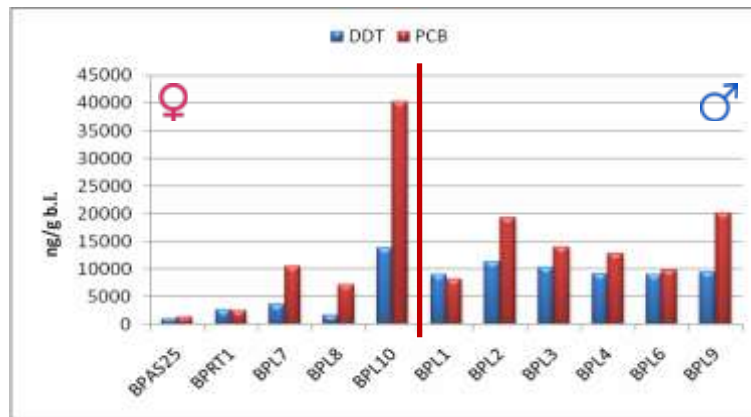


Fig.32. Livelli di organoclorurati (PCB totali – DDT e metaboliti) analizzati per singolo esemplare di balenottera comune del Santuario Pelagos. La linea rossa divide gli esemplari di sesso femminile da quello maschile.

- Livelli di CYP1A1 e CYP2B

L'espressione proteica del CYP1A1 risulta più elevata nei campioni BPL8 e BPL9 (Mar Ligure), mentre il valore più basso è stato evidenziato nell'esemplare di balenottera comune campionata nell'Arcipelago Toscano (BPRT1). Per quanto concerne l'isoforma CYP2B, i livelli di espressione proteica più alti sono stati evidenziati negli esemplari BPRT1 e BPL3 mentre BPL1 e BPL5 mostrano i livelli più bassi. Inoltre, risulta interessante che l'unico esemplare che presenta una espressione proteica del CYP2B più alta del CYP1A1 è l'esemplare BPRT1 (Fig. 33).

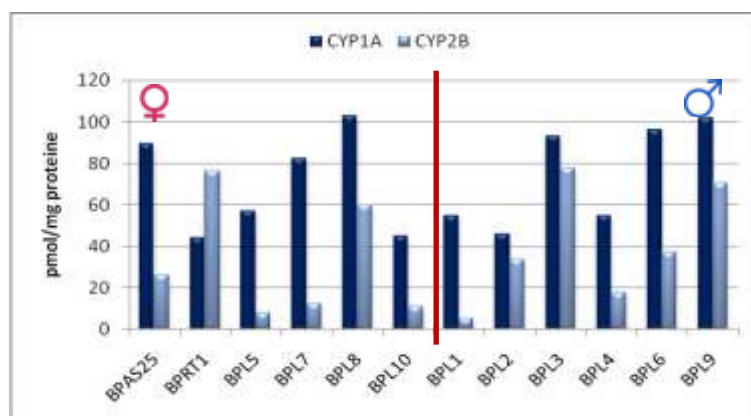


Fig.33. Livelli proteici del CYP1A e CYP1B per singolo esemplare di balenottera comune del Santuario Pelagos. La linea rossa divide gli esemplari di sesso femminile da quello maschile.

- Livelli di Perossidazione Lipidica

I dati riguardanti la perossidazione lipidica, biomarker di stress ossidativo, evidenziano che BPAS25 e BPL3 presentano i livelli più alti rispetto agli altri esemplari (Fig. 34).

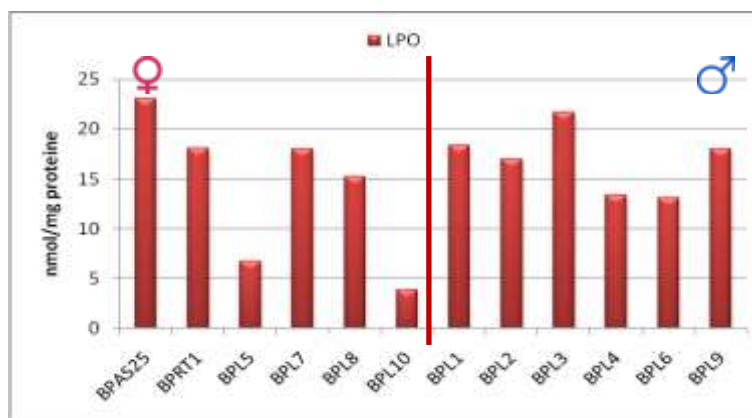


Fig.34. Livelli di perossidazione lipidica (LPO) per singolo esemplare di balenottera comune del Santuario Pelagos. La linea rossa divide gli esemplari di sesso femminile da quello maschi.

- Livelli di espressione genica

L'analisi per ogni individuo analizzato per i cinque geni mette in evidenza la variabilità inter-individuale nella risposta (Fig. 35). L'espressione più elevata per il gene PPARG risulta nell'individuo BPL10 (individuo che presenta peraltro i livelli più alti di OC), mentre la più bassa in BPL 9 con una induzione di 8,5 volte. Per gli individui campionati nel Mar di Sardegna e nell'Arcipelago Toscano, i valori risultato mediamente bassi, circa 1,5 volte in meno rispetto alla media degli individui del Mar Ligure.

Analogamente, anche per i valori di espressione genica del gene PPAR A, gli individui con la più elevata espressione (BPL 8) e la più bassa (BPL2) presentano una over-espressione del gene in BPL 8 di 5 volte rispetto a BPL2, mentre gli individui campionati nelle altre aree di interesse hanno un'espressione mediamente uguale (1,2 volte) rispetto al set di campioni prelevati in Mar Ligure.

Per quanto riguarda le variazioni dei livelli di espressione del CYP1A, la balenottera comune campionata nell'Arcipelago Toscano mostra un'espressione del gene molto elevata, circa 17 volte più elevata rispetto al valore di espressione più basso dell'individuo ligure BPL 9 e 4,9 volte più elevato rispetto alla media degli individui del Mar Ligure. Per quanto riguarda l'espressione del CYP1A, confrontando Mar di Sardegna e Arcipelago Toscano, l'espressione del gene è più elevata di circa 2,5 volte.

I livelli di espressione del gene ER1 sono più elevati nell'individuo BPL8 di 12,6 volte rispetto a BPL10 che presenta espressione più bassa. I valori medi di espressione delle balenottere comuni campionate nel Mar Ligure sono più elevati che nel Mar di Sardegna e l'Arcipelago Toscano rispettivamente di 2 e 1,4 volte.

Infine, l'espressione del gene AhR risulta piuttosto omogenea tra tutti i campioni di *B. physalus*. L'espressione più elevata è risultata nel campione BPL 8, nel quale l'AhR risulta indotto 1,8 volte

rispetto al campione BPL 4. Le differenze tra la media degli individui campionati nel Mar Ligure e quelli del Mar di Sardegna e Arcipelago Toscano è molto bassa (1,2 volte).

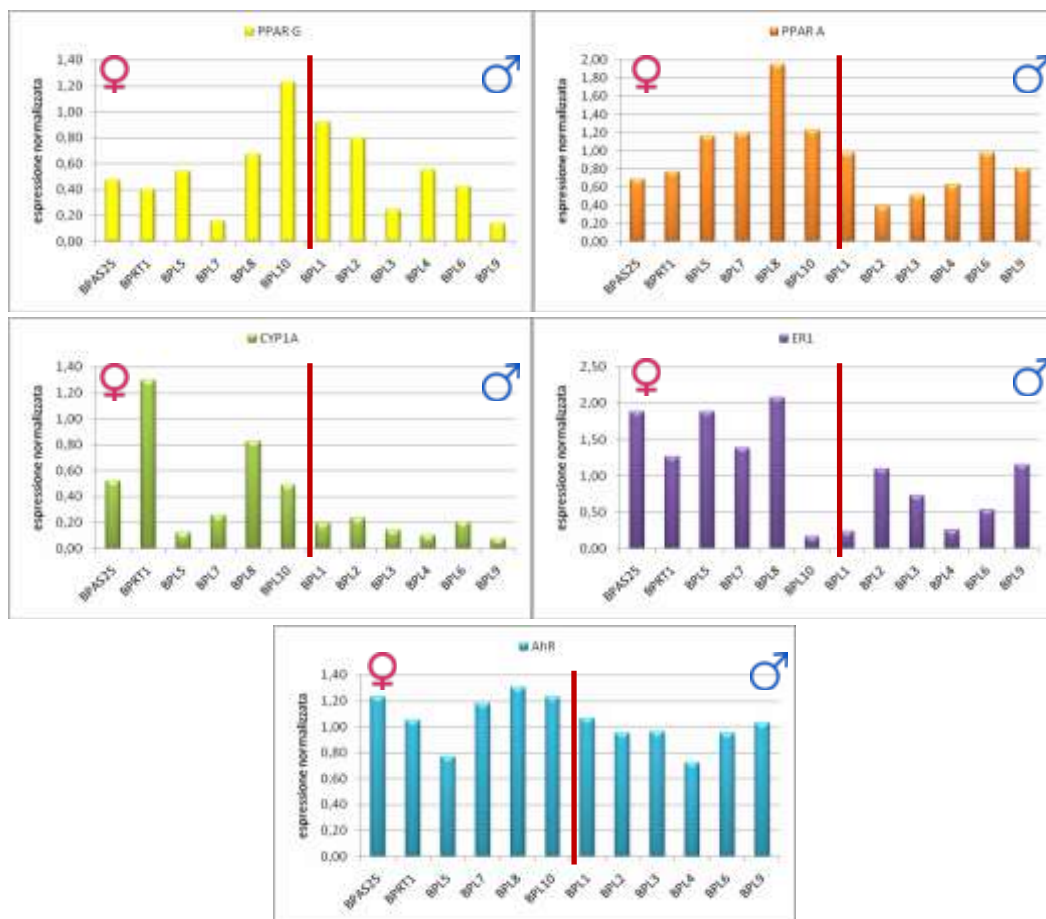


Fig.35. Livelli di espressione genica dei geni PPAR G, PPAR A, CYP1A, ER1 e AhR per singolo esemplare di balenottera comune campionate nel Santuario Pelagos. La linea rossa divide gli esemplari femmine dai maschi.

c) Analisi complessiva 2012 vs 2013 di *B. physalus*

Nei due anni di campionamento nel Mar Ligure sono stati campionati 19 esemplari di balenottera comune di cui 9 nel 2012 (F=4; M=5) e 10 nel 2013 (F=4; M=6).

- Analisi dei contaminanti

Livelli di ftalati

L'analisi dei livelli di MEHP nelle balenottere comuni campionate sia nel Mar di Sardegna che nel Mar Ligure, mettono in evidenza una variazione stagionale di questo composto soprattutto per quanti riguarda gli individui di sesso maschile. I livelli di questo ftalato, infatti, aumentano con un trend temporale da luglio a settembre (Fig. 36).

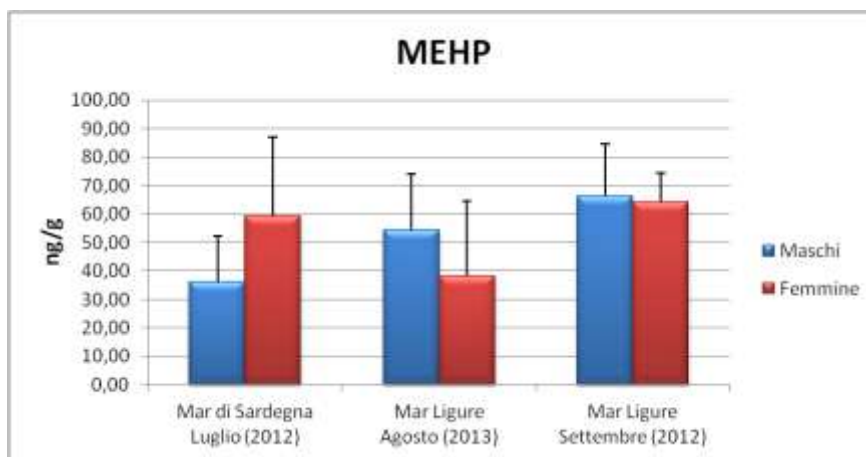


Fig.36. Livelli medi di MEHP (ng/g peso secco) in esemplari maschi e femmine di balenottera comune nel Mar Ligure e nel Mar di Sardegna in diversi periodi del 2012 e 2013.

Livelli degli organoclorurati

Per entrambe le categorie di OC indagate le concentrazioni più elevate dei contaminanti sono state evidenziate negli esemplari di sesso maschile campionati nel 2012, mentre per gli individui di sesso femminile il valore più elevato dei PCB è da attribuirsi ad i valori particolarmente elevati di un singolo individuo (BL10). La differenza nelle concentrazioni nei due anni può essere attribuita al diverso periodo di campionamento, settembre nel 2012 e inizio agosto 2013, e di conseguenza ad una maggiore bioaccumulo di composti liposolubili legato alla dieta negli individui campionati alla fine del periodo estivo di foraggiamento (Fig. 37).

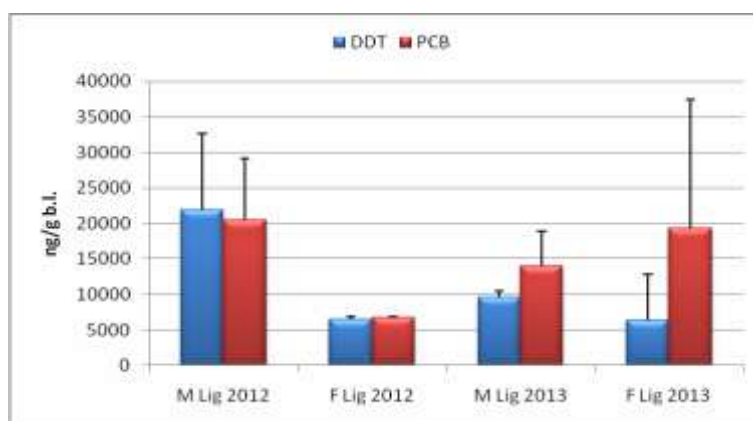


Fig.37. Livelli medi di organoclorurati (PCB totali – DDT e metaboliti) in esemplari maschi e femmine di balenottera comune nel Mar Ligure campionati nel 2012 e 2013.

- Risultati risposte biologiche (biomarkers)

Solo per l'area di campionamento della Liguria è stato possibile effettuare un confronto tra gli individui maschi e femmine campionate nei due diversi anni.

-Livelli di CYP1A1 e CYP2B

Per entrambe le isoforme del citocromo P450 la maggiore induzione proteica è stata evidenziata negli esemplari di sesso maschile senza evidenziare però in entrambi i citocromi differenze statisticamente significative nella variabile sesso. Nella figura 35 è evidente come il valore del CYP2B valutato negli esemplari femminili campionati nel 2013 sia inferiore a quello delle femmine campionate nel 2012 (Fig. 38).

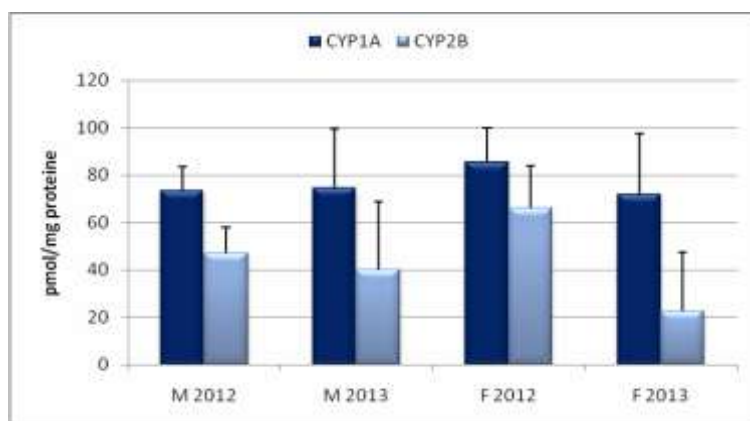


Fig.38. Livelli medi di espressione proteica del CYP1A1 e CYP2B in esemplari maschi e femmine di balenottera comune del Santuario Pelagos in due diversi anni di campionamento.

- Livelli di Perossidazione Lipidica

I dati riguardanti la perossidazione lipidica, biomarker di stress ossidativo, evidenziano un valore leggermente maggiore degli individui di sesso maschile rispetto ai maschi campionati nel 2013 mostrando una differenza statisticamente significativa (Fig. 39).

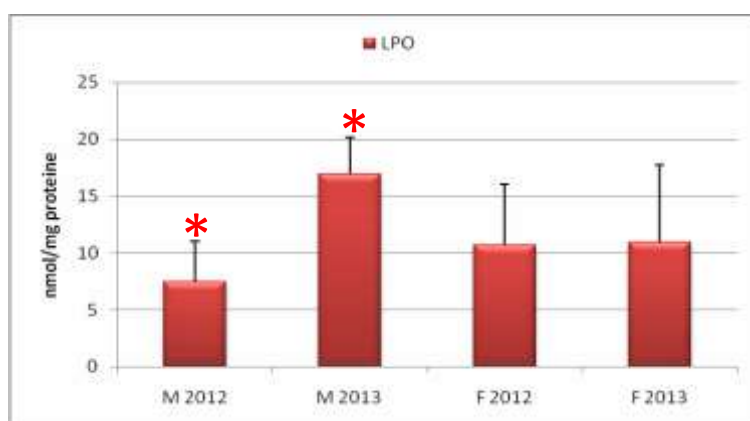


Fig.39. Confronto dei livelli medi di perossidazione lipidica in esemplari maschi e femmine di balenottera comune del Santuario Pelagos campionati nel 2012 e 2013. * indica differenze statisticamente significative ($p < 0.05$) tra gli individui di sesso maschile negli anni 2012 e 2013.

- Livelli di espressione genica

L'espressione dei quattro geni biomarkers è stata messa a confronto per valutare le possibili variazioni negli anni 2012 e 2013 tra gli esemplari di balenottera comune campionati nella medesima area del Santuario Pelagos (Fig. 40). Il gene PPAR G, nel 2012 presenta valori di mRNA più elevati sia per i maschi che per le femmine, mentre per il gene PPAR A risulta un andamento opposto.

Nell'anno 2012 gli individui di balenottera comune, sia maschi che femmine, presentano dei livelli di espressione del CYP1A più elevati (le femmine di circa 10 volte) rispetto ai valori di espressione dello stesso gene negli individui campionati nel 2013. Questo dato è strettamente correlato alle diverse concentrazioni di contaminanti OC rilevate nei due anni/periodi di campionamento.

Per quanto riguarda il gene ER 1, invece, l'espressione più elevata risulta nelle femmine campionate nel 2013 e nei maschi campionati nel 2012. L'espressione del gene AhR appare moderatamente più elevata sia nei maschi che nelle femmine di balenottera comune campionate nel 2013 rispetto al 2012.

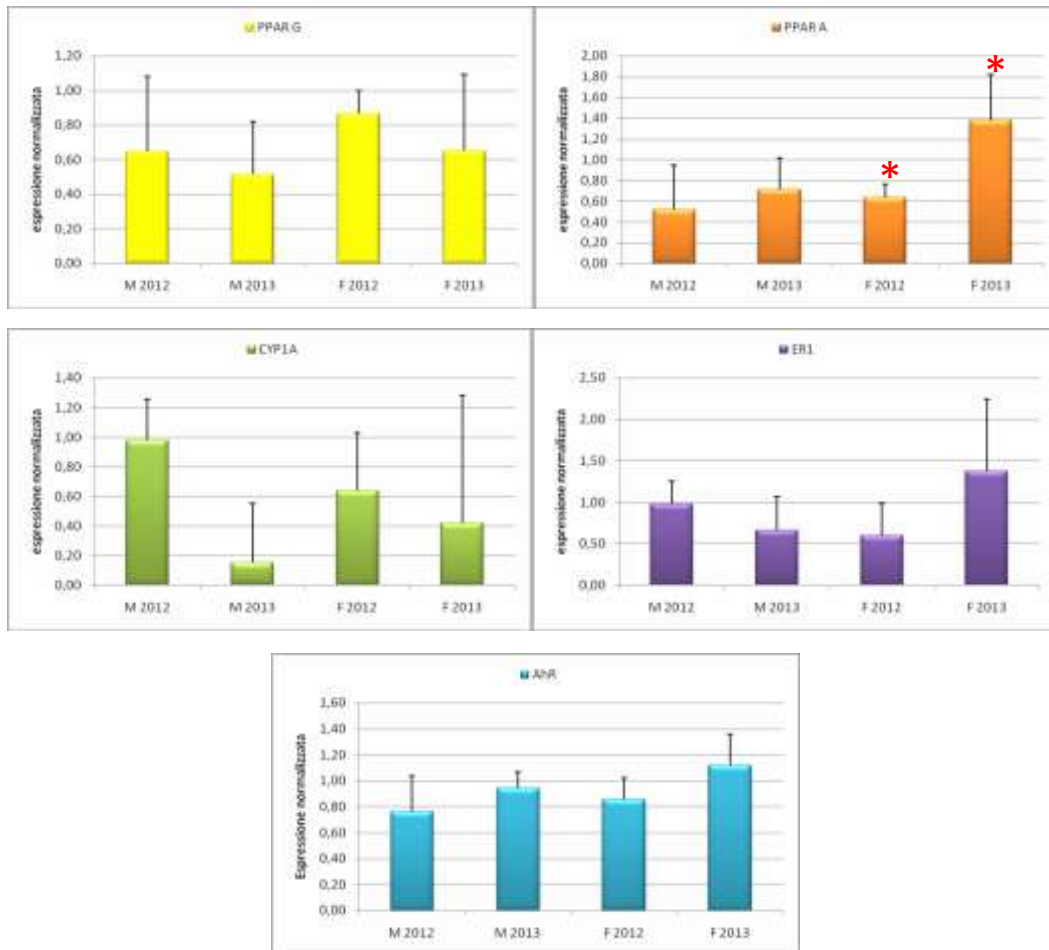


Fig.40. Confronto dei livelli medi di espressione dei geni PPAR G, PPAR A, CYP1A, ER1 ed AhR in esemplari maschi e femmine di balenottera comune del Santuario Pelagos campionate nel 2012 e 2013. * indica differenze statisticamente significative ($p < 0,05$).

4) **RISULTATI** DELLE DIAGNOSI ECOTOSSICOLOGICHE SUL POTENZIALE IMPATTO DELLE MICROPLASTICHE E DERIVATI TOSSICOLOGICAMENTE ATTIVI SU *S. COERULEOALBA* CAMPIONATA NELLE AREE DEL SANTUARIO PELAGOS.

a) **Analisi complessiva Maschi vs Femmine**

- **Analisi dei contaminanti - Livelli degli organoclorurati**

Per entrambe le categorie di OC indagate le concentrazioni più elevate dei contaminanti sono state evidenziate negli esemplari di sesso maschile senza differenze statisticamente significative (Fig. 41).

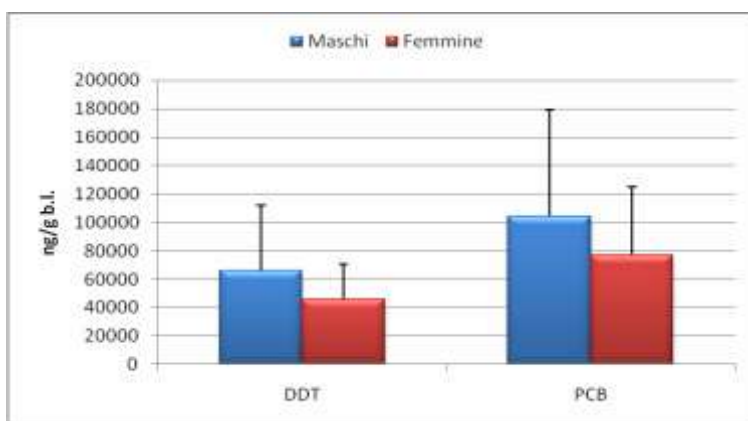


Fig.41. Livelli medi di OC (DDT – PCB totali) in esemplari maschi (n=5) e femmine (n=5) di stenella striata campionate nel Santuario Pelagos.

- **Livelli di CYP1A1 e CYP2B**

Per entrambe le isoforme del citocromo P450 la maggiore induzione proteica è stata evidenziata negli esemplari di sesso femminile nonostante non vi siano differenze statisticamente significative (Fig. 42).

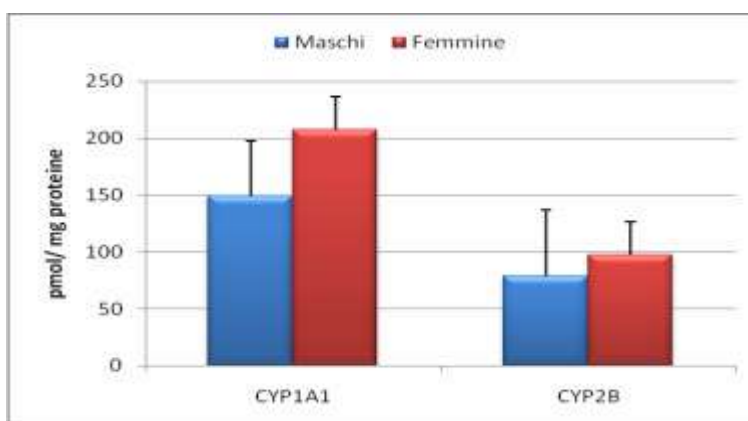


Fig.42. Livelli proteici di medi CYP1B e CYP2B in esemplari maschi (n=5) e femmine (n=11) di stenella striata campionata del Santuario Pelagos.

- Livelli di Perossidazione Lipidica

I dati riguardanti la perossidazione lipidica, biomarker di stress ossidativo, confermano le evidenze ottenute con l'analisi delle isoforme del citocromo; un valore moderatamente maggiore negli individui femmine rispetto ai maschi (Fig. 43).

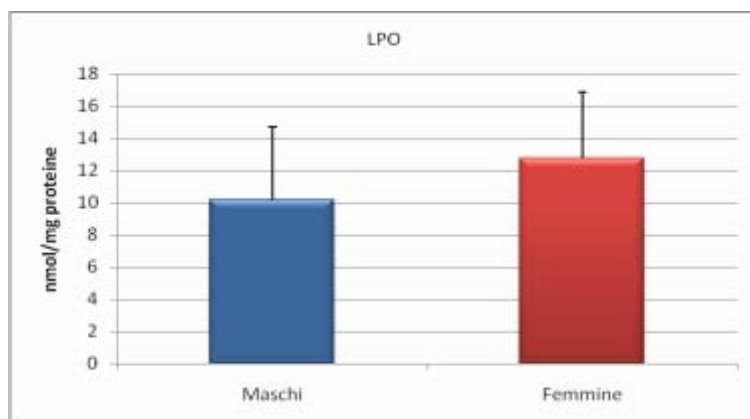


Fig.43. Livelli di perossidazione lipidica in esemplari maschi (n=5) e femmine (n=11) di stenella striata campionata del Santuario Pelagos.

- Livelli di espressione genica

L'espressione dei geni PPAR G, PPAR A, ER 1, CYP1A e AhR è stata misurata negli esemplari di stenella striata campionati nel Santuario Pelagos. Dal confronto tra gli individui maschili e femminili è evidente come l'espressione dei geni PPAR G e AhR sia pressochè uguale tra maschi e femmine. Gli individui di sesso femminile presentano, invece, espressioni più elevate dei geni PPAR A e ER 1. Al contrario l'espressione del gene CYP1A sembra subire una *over*-espressione negli individui di sesso maschile (Fig. 44).

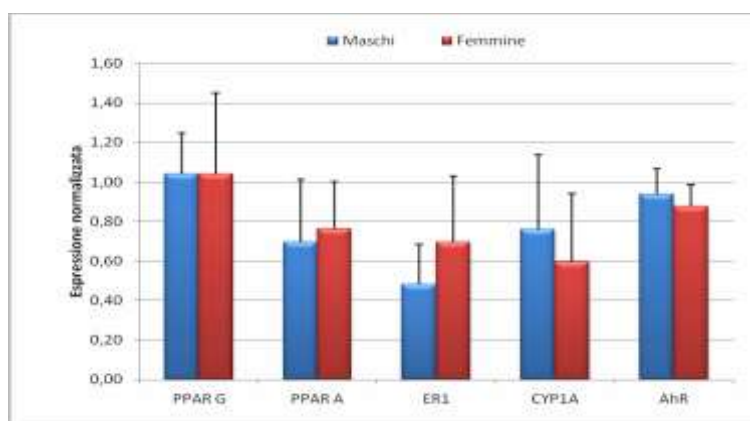


Fig.44. Livelli di espressione genica dei geni PPAR G, PPAR A, ER1, CYP1A ed AhR in esemplari maschi (n=5) e femmine (n=10) di stenella striata campionata del Santuario Pelagos.

b) Analisi dei livelli di contaminanti e delle risposte dei biomarker per singolo campione di *S. coeruleoalba*

- Analisi dei contaminanti - Livelli degli organoclorurati

Analizzando i livelli di PCB e DDT per singolo campione di stenella striata, AST14 e AST31 presentano i valori più elevati, mentre i livelli di DDT sono più elevati nel campione AST34 e AST27. Al contrario, i valori più bassi sia per i PCB e DDT sono stati misurati nel campione AST19 (Fig. 45).

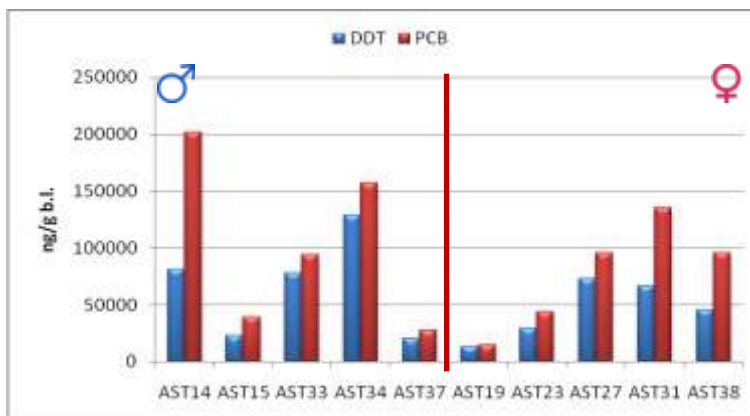


Fig.45. Livelli di organoclorurati (PCB totali – DDT) analizzati per singolo esemplare di stenella striata del Santuario Pelagos. La linea rossa divide gli esemplari di sesso maschile dalle femmine.

c) Risposte di Biomarkers

- Livelli di espressione proteica CYP1A1 e CYP2B

L'espressione proteica del CYP1A1 è risultata più elevata nei campioni di sesso femminile AST12, AST16 ed AST25 (Mar di Sardegna), mentre il valore più basso negli esemplari di sesso maschile AST24 ed AST35 (Mar di Sardegna). Per quanto concerne l'isoforma CYP2B, i livelli di espressione proteica più alti risultano nell'esemplare IST210 (Mar Ligure) mentre il valore più basso risulta nell'individuo di sesso maschile AST35.

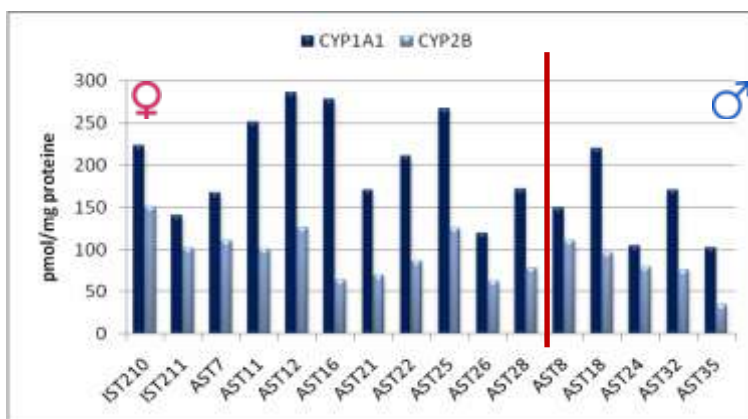


Fig.46. Livelli proteici di medi CYP1B e CYP2B in esemplari maschi (n=5) e femmine (n=11) di stenella striata campionata del Santuario Pelagos. La linea rossa divide gli esemplari femmine da quelli di sesso maschile.

- Livelli di perossidazione lipidica

L'esemplare AST16 per le femmine e AST 32 (Mar di Sardegna) per i maschi mostrano i livelli più elevati di perossidazione lipidica. Tuttavia la variabilità inter-individuale risulta piuttosto bassa sia nei maschi che nelle femmine (Fig. 47).

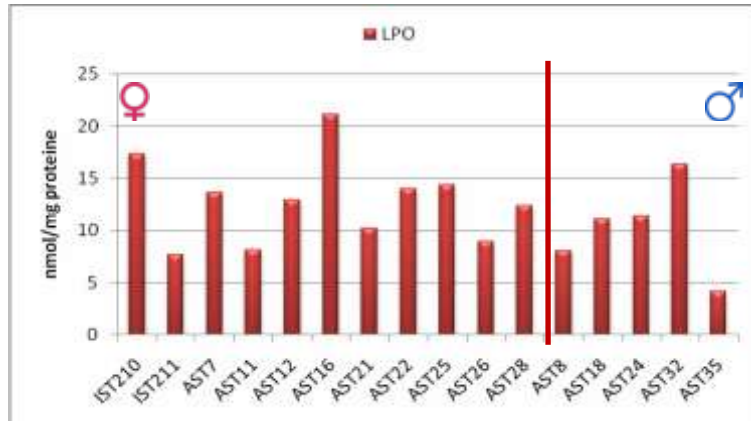


Fig.47. Livelli di perossidazione lipidica in esemplari maschi (n=5) e femmine (n=11) di stenella striata campionata del Santuario Pelagos. La linea rossa divide gli esemplari femmine da quelli di sesso maschile.

- Livelli di espressione genica:

L'espressione del gene PPARG risulta più elevata negli individui di sesso femminile campionati nel Mar Ligure (IST 210 e 211), mentre i valori più bassi sono stati ottenuti per gli individui femminili AST 7 e AST 28. Il gene PPAR A è maggiormente espresso nei campioni di stenelle femmine AST11, AST 22 e IST210, e negli individui di sesso maschile AST8 e AST24. L'espressione CYP1A è più elevata negli individui maschili AST8 e AST35, che se confrontata all'espressione del CYP1A nel campione AST16 è 8 volte maggiore. L'espressione del gene ER1 risulta più elevata di 8.2 volte nel campione AST12 rispetto al campione IST 211. I maschi presentano espressioni moderatamente elevate, nonostante che il recettore sia strettamente legato agli ormoni femminili. Per il gene AhR l'espressione è mediamente omogenea in tutti gli individui sia di sesso femminile che maschile (Fig. 48)

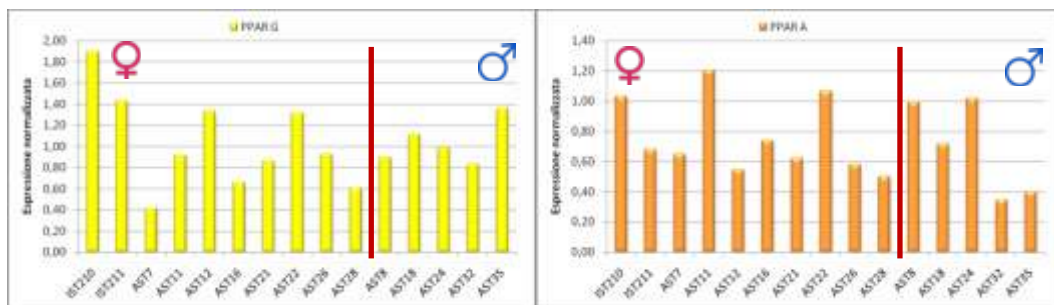




Fig.48. Livelli di espressione genica dei geni PPAR G, PPAR A, CYP1A, ER1 ed AhR in esemplari maschi (n=5) e femmine (n=10) di stenella striata campionata del Santuario Pelagos. La linea rossa divide gli esemplari femmine da quelli di sesso maschile.

e) Analisi delle correlazioni

Interessanti correlazioni sono state evidenziate fra l'induzione del CYP1A1 e l'up-regulation del recettore AhR nelle femmine di stenella striata. Questo dato conferma la stretta dipendenza della risposta del CYP1A all'attivazione da parte del recettore Ah e il loro ruolo come indicatori dell'esposizione a contaminanti liposolubili (Fig. 49).

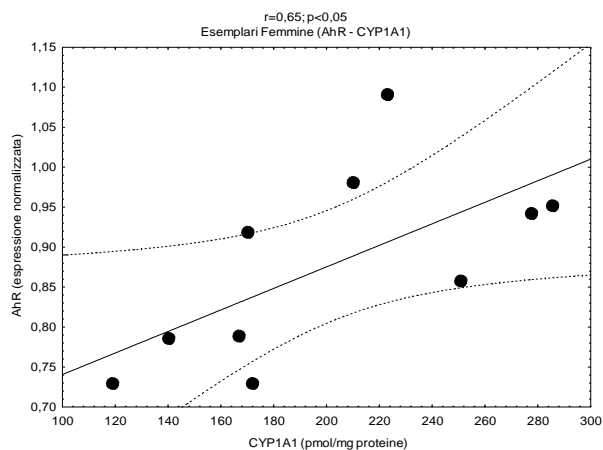


Fig.49. Correlazioni fra i Livelli di espressione genica dei CYP1A e AhR in esemplari femmine (n=10) di stenella striata campionata del Santuario Pelagos.

Una seconda correlazione è stata evidenziata fra i livelli di perossidazione lipidica e l'up-regulation del recettore Ah, dovuta probabilmente all'esposizione a stress ambientali, in particolare composti diossino-simili.

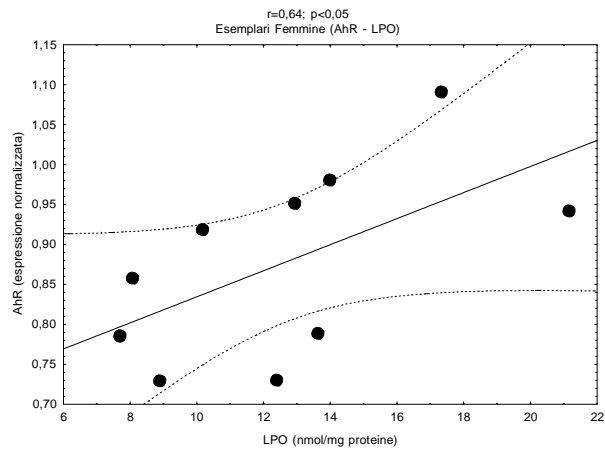


Fig.50. Correlazioni fra i livelli di espressione genica del AhR ed i livelli di LPO in esemplari femmine (n=10) di stenella striata campionata del Santuario Pelagos.

5) RISULTATI DELLE DIAGNOSI ECOTOSSICOLOGICHE SUL POTENZIALE IMPATTO DELLE MICROPLASTICHE E DERIVATI TOSSICOLOGICAMENTE ATTIVI SU ALTRE SPECIE DI CETACEI (*Delphinus delphis*, *Grampus griseus* e *Tursiops truncatus*) CAMPIONATE NELLE AREE DEL SANTUARIO PELAGOS.

Analisi dei Contaminanti - Livelli degli organoclorurati

Per entrambe le categorie di OC indagate le concentrazioni più elevate dei contaminanti sono stata evidenziate in un esemplare di delfino comune campionato nel Mar di Sardegna. Il ridotto numero di campioni per specie non consente però un'adeguata analisi statistica (Fig. 51).

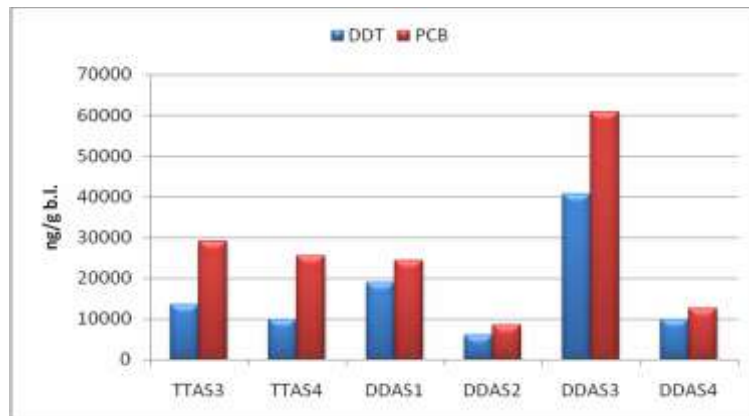


Fig.51. Livelli di organoclorurati (DDT e metaboliti – PCB totali) per singolo esemplare di tursiopo (TTAS3-4) e delfino comune (DDAS1-4) campionati nel Santuario Pelagos.

- Livelli di espressione proteica del CYP1A1 e CYP2B

Per entrambe le isoforme di citocromo indagate, le concentrazioni più elevate sono state evidenziate nell'unico individuo di grampo campionato. I livelli proteici del CYP1A1 e CYP2B nella specie delfino comune, invece, risultano più bassi negli individui che presentano le concentrazioni dei contaminanti OC più elevati (Fig. 51-52).

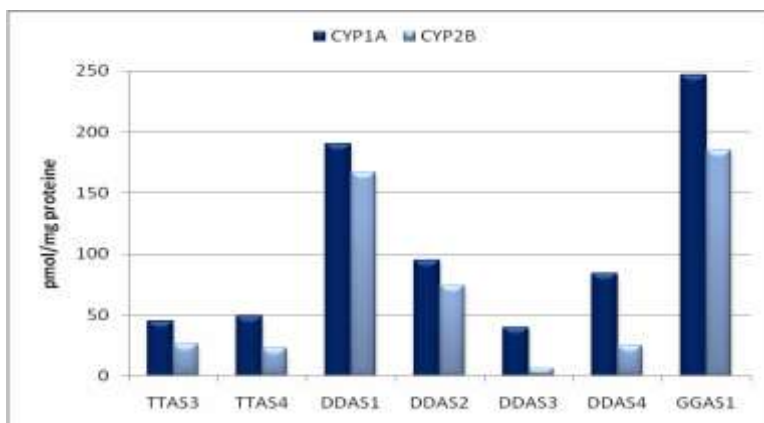


Fig.52. Livelli di espressione proteica del CYP1A1 e CYP2B per singolo esemplare di tursiopo (TTAS3-4), delfino comune (DDAS1-4) e grampo (GGAS1) campionati nel Santuario Pelagos.

- Livelli di perossidazione lipidica

I valori di perossidazione lipidica più elevati sono stati evidenziate nel campione DDAS4 che tuttavia presenta livelli di OC più bassi insieme a DDAS2. Il ridotto numero di campioni per specie non consente però un'adeguata analisi statistica (Fig. 53).

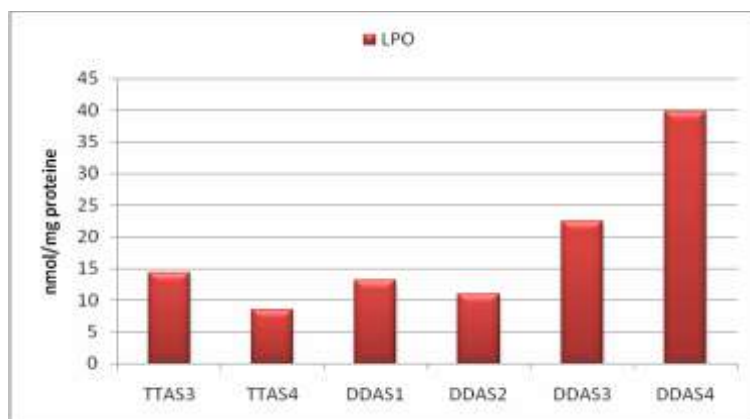


Fig.53. Livelli di perossidazione lipidica per singolo esemplare di tursiopo (TTAS3-4) e delfino comune (DDAS1-4) campionati nel Santuario Pelagos.

6) RISULTATI DELLE DIAGNOSI ECOTOSSICOLOGICHE SUL POTENZIALE IMPATTO DELLE MICROPLASTICHE E DERIVATI TOSSICOLOGICAMENTE ATTIVI SU QUATTRO SPECIE DI CETACEI CAMPIONATE NELLE AREE DEL SANTUARIO PELAGOS.

Un'analisi complessiva dei contaminanti e dell'espressione proteica comparando tutte le specie campionate nelle campagne del 2013 è stata effettuata al fine di individuare il diverso impatto delle microplastiche e dei derivati tossicologicamente attivi a livello inter-specifico.

- Analisi dei Contaminanti - Livelli degli organoclorurati

Le concentrazioni dei contaminanti organoclorurati nelle quattro specie analizzate sia odontoceti (delfino comune, tursiope, stenella striata) che misticeti (balenottera comune) risultano più elevate nella specie stenella striata, confermando l'elevata esposizione di questa specie a composti liposolubili assunti tramite la dieta. Le concentrazioni più basse sia di PCB totali che di DDT sono stati misurati nel blubber sottocutaneo delle biopsie di balenottera comune (Fig. 54).

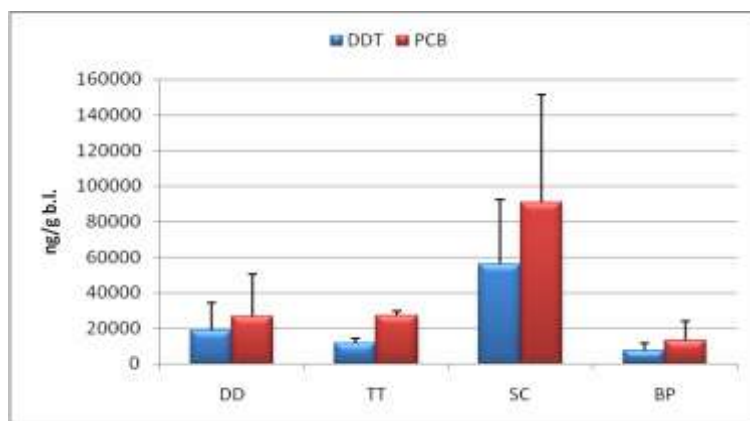


Fig.54. Livelli organoclorurati (PCB totali – DDT e metaboliti) nelle quattro specie di cetacei campionate nel Santuario Pelagos nell'anno 2013. DD (*D. delphis*), TT (*T. truncatus*), SC (*S. coeruleoalba*) e BP (*B. physalus*).

- Livelli di espressione proteica del CYP1A1 e CYP2B e di perossidazione lipidica

Le risposte biomarkers per l'espressione proteica CYP1A1 e CYP2B analizzate nelle quattro specie hanno evidenziato livelli di induzione proteica più elevati nella specie stenella striata come confermato anche dalle concentrazioni dei contaminanti organoclorurati (Fig. 54). La balenottera, pur avendo i livelli di PCB e Oc più bassi, presenta comunque una induzione delle due isoforme nell'ordine di grandezza delle altre due specie di odontoceti analizzate (tursiope e delfino comune). I livelli di perossidazione lipidica, invece, risultano omogenei nelle quattro specie, ma le risposte più elevate sono stati riscontrati nel delfino comune.

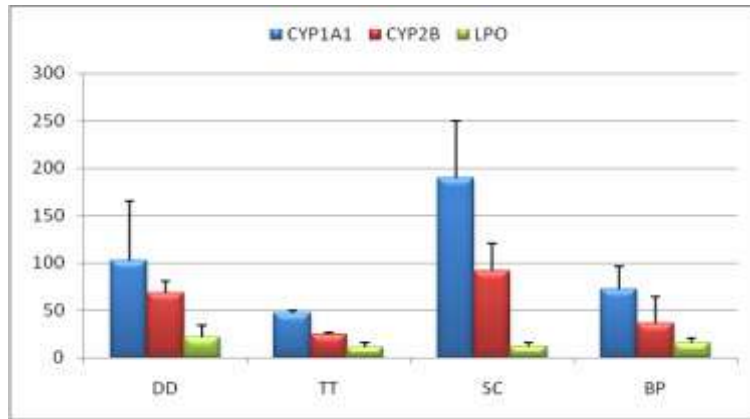


Fig.55. Livelli di CYP1A1, CYP2B e LPO in quattro specie di cetacei campionati nel 2013 nel Santuario Pelagos. (*D. delphis*), TT (*T. truncatus*), SC (*S. coeruleoalba*) e BP (*B. physalus*). I CYP1A1 e 2B sono espressi in pmol/mg proteine, i valori di LPO in nmol/mg proteine.

E) SVILUPPO DI TECNICHE DI MICROSCOPIA OTTICA ED ELETTRONICA A SCANSIONE E TRASMISSIONE PER LO STUDIO ANATOMICO E L'EVENTUALE INDIVIDUAZIONE DI MICROPLASTICHE IN EUFASIACEI CAMPIONATI NEL SANTUARIO PELAGOS

Attraverso l'utilizzo del SEM (scanning electron microscope) e del TEM (trasmission electron microscope) è stata descritta l'anatomia del tratto gastrointestinale di esemplari di eufasiacei campionati nelle tre aree di studio del Santuario Pelagos per ottenere un quadro anatomicamente completo di questa specie al fine di valutare l'eventuale presenza di microplastiche ingerite. I campioni sono stati fissati immediatamente dopo il prelievo per la successiva analisi tramite entrambe le tipologie di microscopio. Una volta messa a punto e validata la migliore procedura attraverso prove sperimentali (diversi tempi di fissazione, diversi fissativi e diverse concentrazioni dei tamponi utilizzati) svolte su differenti specie di eufasiacei, è stato necessario il reperimento dei campioni di eufasiacei che riguardavano più da vicino il tema dell'impatto sulla balenottera concentrandosi sulla sua principale preda: la *Meganictyphanes norvegica*. L'analisi dei campioni di dimensioni tra i 5 e i 6 cm di questa specie è stata dipendente alla difficile reperibilità di organismi di tali dimensioni che risultano fondamentali per l'analisi del tratto gastrointestinale. I primi campioni ottenuti attraverso le retinate notturne/diurne (utilizzati come descritto sopra per la messa a punto della fissazione), essendo di dimensioni inferiori hanno reso difficile o non hanno permesso, in alcuni casi, l'isolamento del tratto gastrointestinale. Tuttavia, gli esemplari campionati nel Santuario Pelagos nel Settembre 2013 hanno reso possibile per la prima volta l'isolamento sia dello stomaco che dell'intestino e la loro fissazione per la successiva visualizzazione microscopio ottico (Fig. 56 e 58) ed al TEM. Inoltre in alcuni campioni è stata possibile la loro visualizzazione al SEM dopo una opportuna suddivisione longitudinale del campione (Fig. 57).

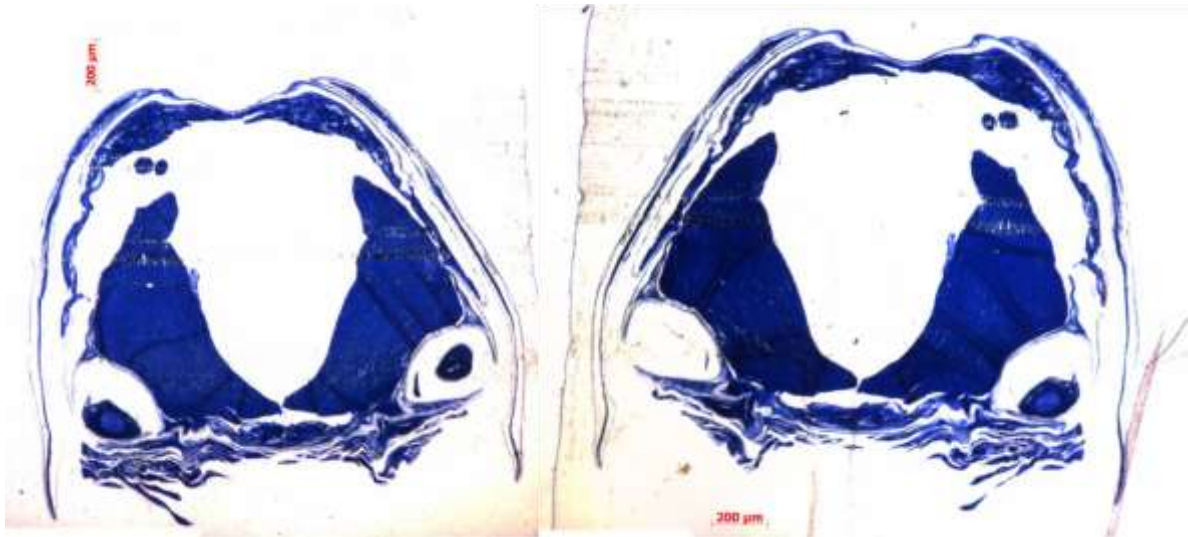


Fig.56. Sezione sagittale del tratto gastrointestinale di esemplari di *Meganictyphanes norvegica* al microscopio ottico.

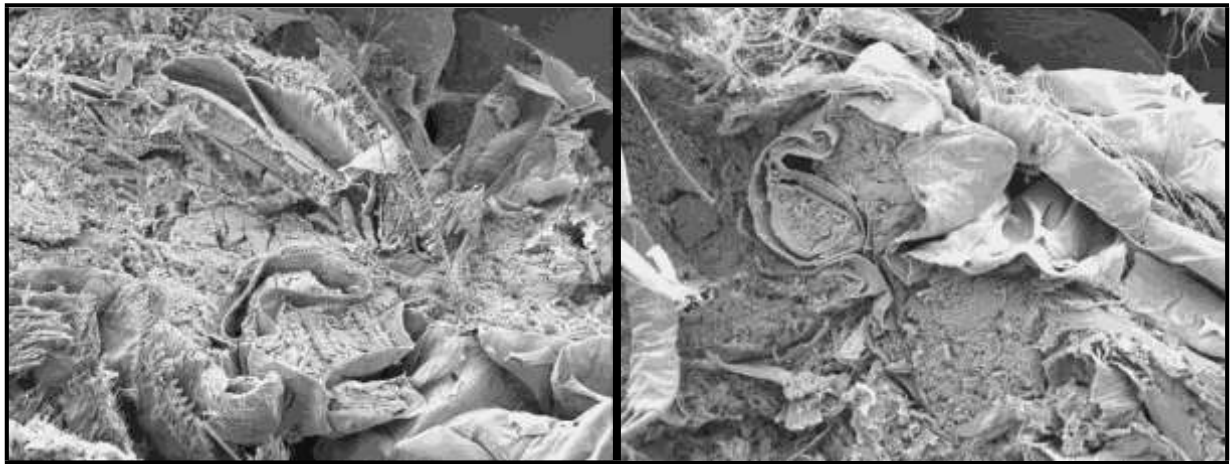


Fig.57. Sezione longitudinale del tratto gastrointestinale di *Meganicthyphanes norvegica* al SEM.

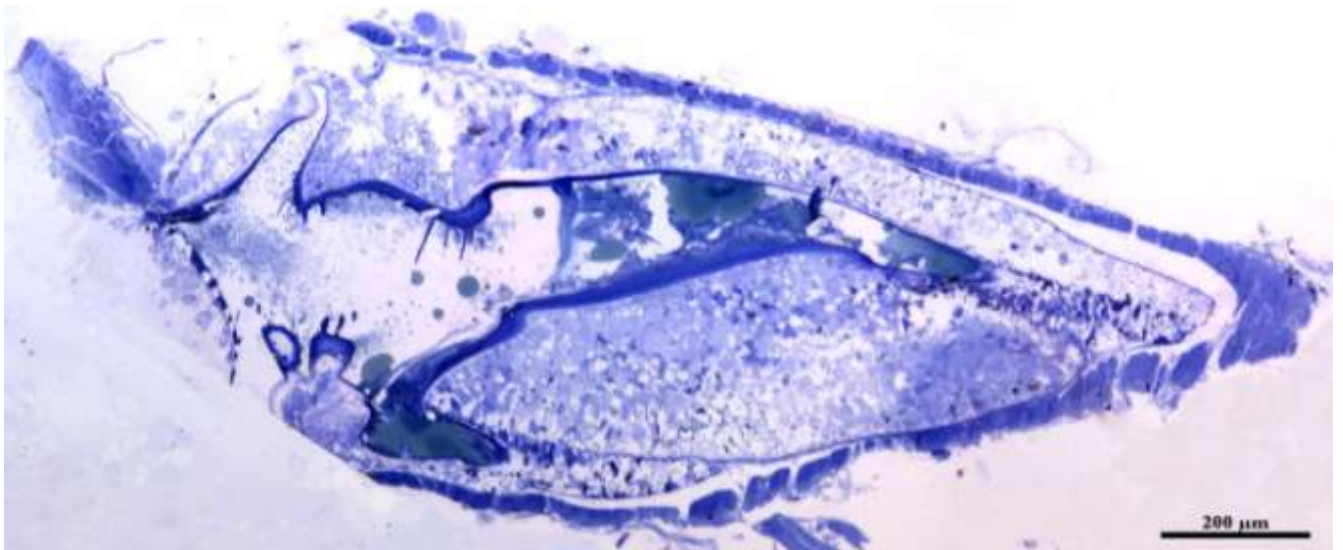


Fig.58. Sezione sagittale dello stomaco di esemplari di *M. norvegica* al microscopio ottico.

E) SVILUPPO DI NUOVI BIOMARKES SU INDIVIDUI FREE-RANGING DI VARIE SPECIE DI CETACEI E BALENOTTERA COMUNE SPECIFICI PER INDIVIDUARE LA PRESENZA E GLI EFFETTI DEGLI ADDITIVI DELLE PLASTICHE (IN PARTICOLARE FTALATI).

Le *slices* (colture organotipiche “ex vivo”) di tessuto cutaneo e sottocutaneo sono state ottenute da sub-aliquote di biopsia cutanea ed esposte seguendo il protocollo descritto da Fossi *et al.* (2010). Le *slices* trattate sono state incubate per 24h in un mezzo di coltura cellulare con concentrazioni diverse delle diverse classi di contaminanti.

- Esperimenti *in vitro* ed *ex-vivo*

Slices di biopsia cutanea di balenottera comune e tursiope sono state esposte a concentrazioni crescenti di composti derivati o associati alla plastica quali bisfenolo A e ftalati, direttamente durante le attività di campionamento.

Sulle *slices* sono state valutate le variazioni di espressione di geni coinvolti nel metabolismo dei composti derivati della plastica per determinare l'esposizione e la risposta degli organismi in successivi studi in natura. I biomarker molecolari analizzati sono: i recettori Peroxisome *proliferator-activated* alpha e gamma (PPAR α e γ), il recettore per gli estrogeni alpha (ER α), il citocromo P450 1A1 (CYP1A) e 2B (CYP2B). In particolare, sono stati selezionati i geni PPAR A e G poiché sono coinvolti nel metabolismo dei composti additivi della plastica. I recettori “*Peroxisome proliferator-activated*” (PPARs) appartengono ad una superfamiglia di recettori ormonali nucleari, di cui sono presenti tre isoforme: PPAR A, B e G. Studi effettuati sui composti perfluorinati suggeriscono che alcuni effetti biologici dei PFC, ftalati e bisfenolo A sono mediati attraverso i PPAR.

Culture organotipiche di balenottera comune: trattamento con miscela di ftalati

Cinque colture organotipiche allestite da biopsie cutanee sono state trattate con una miscela di ftalati a concentrazioni crescenti. *Slices* di biopsie cutanee provenienti da 3 femmine (BPAS 5, BPAS 6, BPL 5) e 2 maschi (BPAS8 e BPAS 12) sono stati trattate con concentrazioni crescenti di una miscela di ftalati (metanolo-controllo, 0,1 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$). Dall'analisi complessiva delle 5 curve emerge un aumento significativo dell'espressione media del gene PPAR G alle dosi più alte del trattamento (Fig. 59). In particolare, analizzando separatamente le curve derivanti da biopsie prelevate da individui maschi e femmine è evidente come l'espressione del gene PPAR G sia diversamente indotto nelle femmine, nelle quali si ha una repressione dovuta alla concentrazione di trattamento più elevata, mentre nei maschi l'espressioni aumenti di circa 22 volte alle concentrazioni più elevate rispetto al controllo. Il gene PPAR A è moderatamente indotto in seguito al trattamento con le dosi 0,1 $\mu\text{g/ml}$ e 1 $\mu\text{g/ml}$ della miscela di ftalati nei maschi, mentre nelle femmine non si ha una relazione lineare tra dose e risposta. Analogamente, anche l'espressione del gene ER 1 non mostra una linearità dose-risposta in entrambi i sessi. (Fig. 60-61)

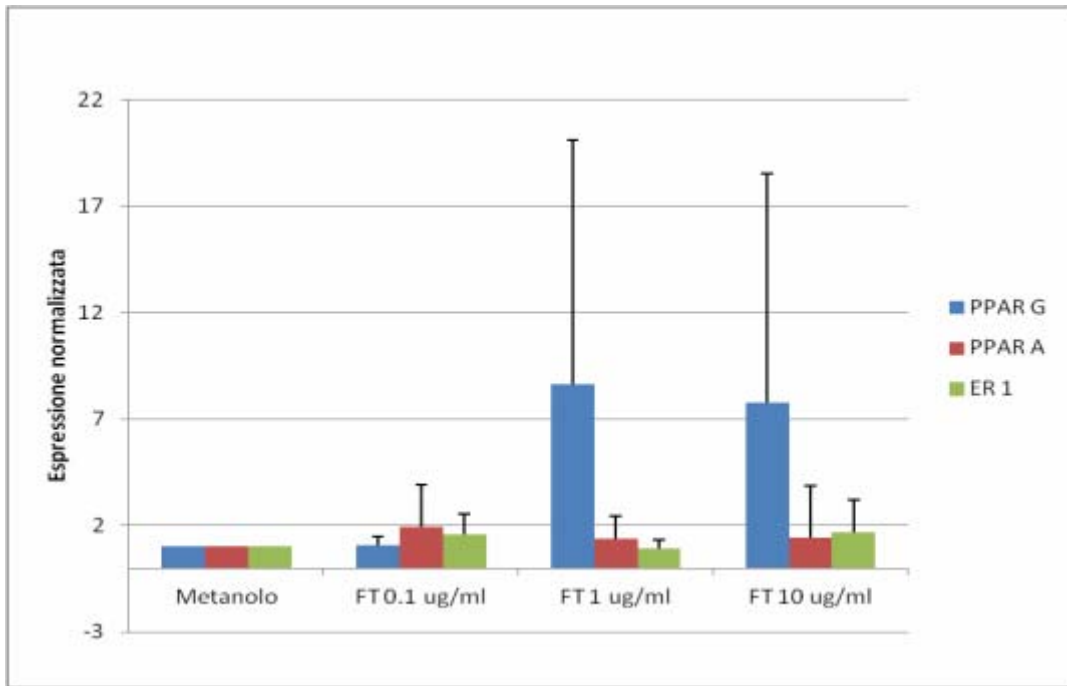


Fig.59. Livelli espressione dei geni PPAR G, PPAR A e ER 1 nei trattamenti ex-vivo in slices di biopsia cutanea di balenottera comune trattate con una miscela di ftalati (FT) a dosi crescenti.

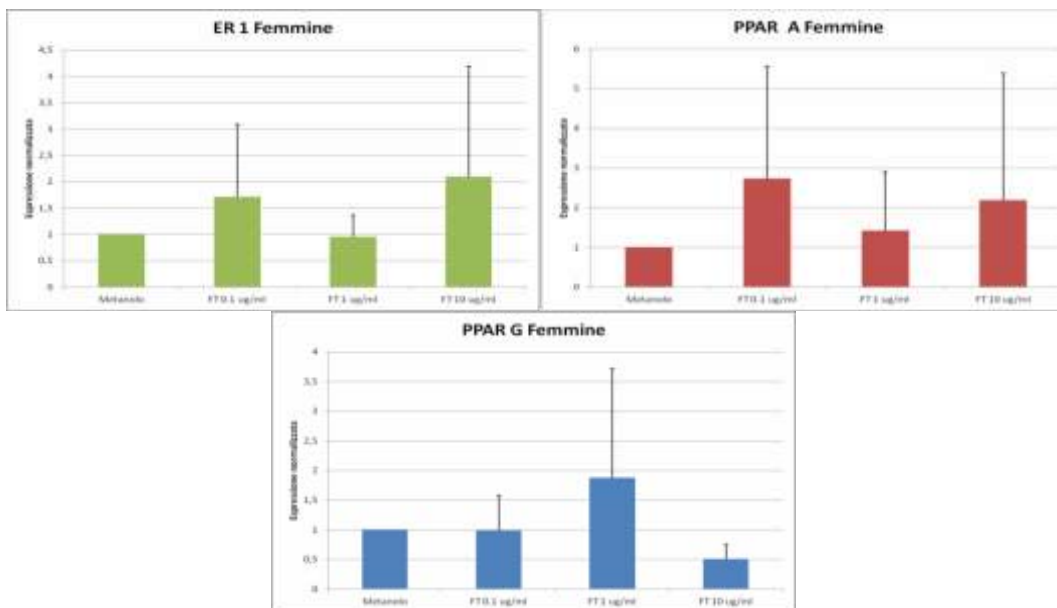


Fig.60. Livelli espressione dei geni PPAR G, PPAR A e ER 1 nei trattamenti ex-vivo in slices di biopsia cutanea di femmine di balenottera comune trattate con una miscela di ftalati (FT) a dosi crescenti.

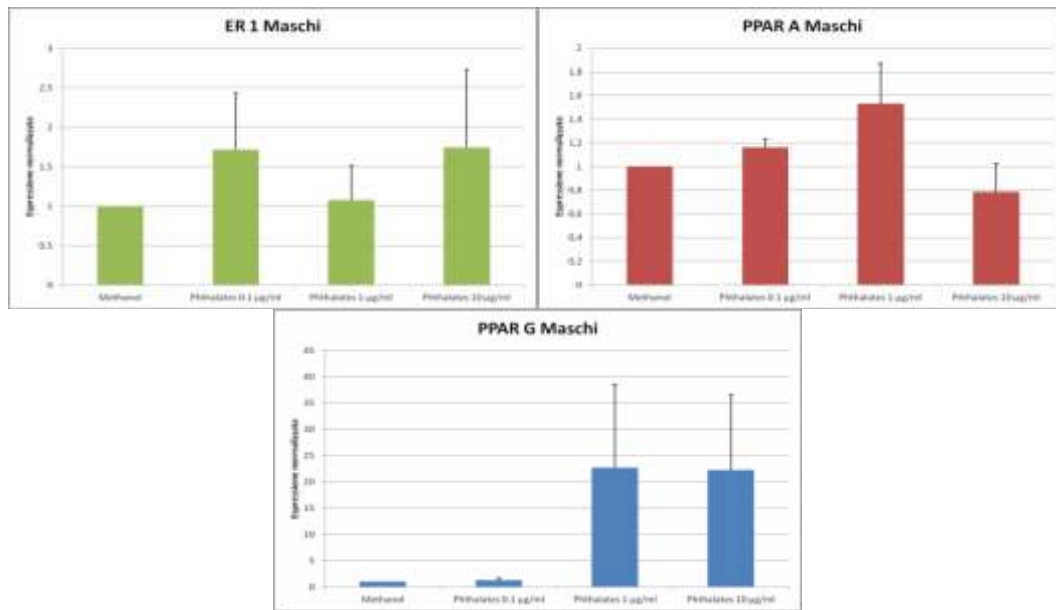


Fig.61. Livelli espressione dei geni PPAR G, PPAR A e ER 1 nei trattamenti ex-vivo in slices di biopsia cutanea di maschi di balenottera comune trattate con una miscela di ftalati (FT) a dosi crescenti.

Colture organotipiche di balenottera comune: trattamento con bisfenolo A

Tre colture organotipiche allestite da biopsie cutanee sono state trattate con concentrazioni crescenti di bisfenolo A (etanolo-controllo; 0,1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml). I trattamenti *ex-vivo* sono stati condotti su due biopsie di balenottera comune femmina (BPAS 6, BPT 21) e un maschio (BPAS12). Dall'analisi complessiva delle tre curve, il gene PPAR A presenta un'espressione media che aumenta con l'aumentare delle dosi di trattamento (1 µg/ml, 10 µg/ml, 100 µg/ml). Al contrario l'espressione dei geni PPAR G e ER 1 non presentano una correlazione dose risposta, in particolare l'espressione è più elevata nel controllo rispetto alle dosi crescenti di bisfenolo A.

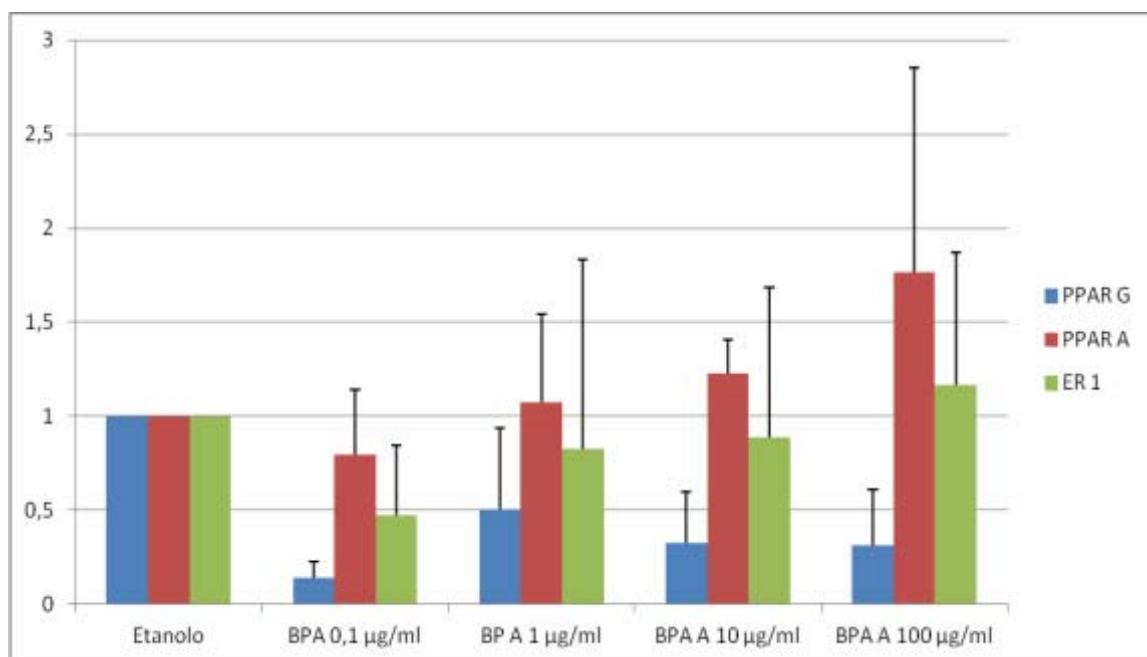


Fig.62. Livelli espressione dei geni PPAR G, PPAR A e ER 1 nei trattamenti ex-vivo in slices di biopsia cutanea di balenottera comune trattate con bisfenolo A (BPA) a dosi crescenti.

Culture organotipiche di tursiope: trattamento con miscela di ftalati

Da un individuo maschio di tursiope sono state allestite colture organotipiche trattate con concentrazioni crescenti di una miscela di ftalati come descritto per la balenottera comune. L'espressione del gene PPAR G presenta un andamento dose-risposta che aumenta all'aumentare della dose di trattamento rispetto al controllo (metanolo). Al contrario, l'espressione del gene PPAR A non mostra alterazioni dei livelli di espressione in seguito all'esposizione alla stessa miscela.

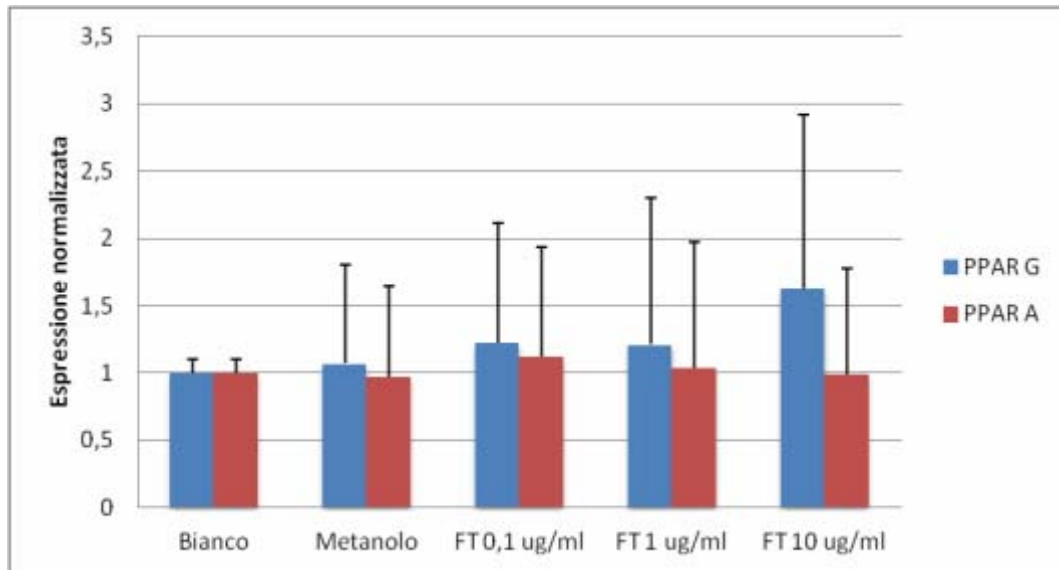
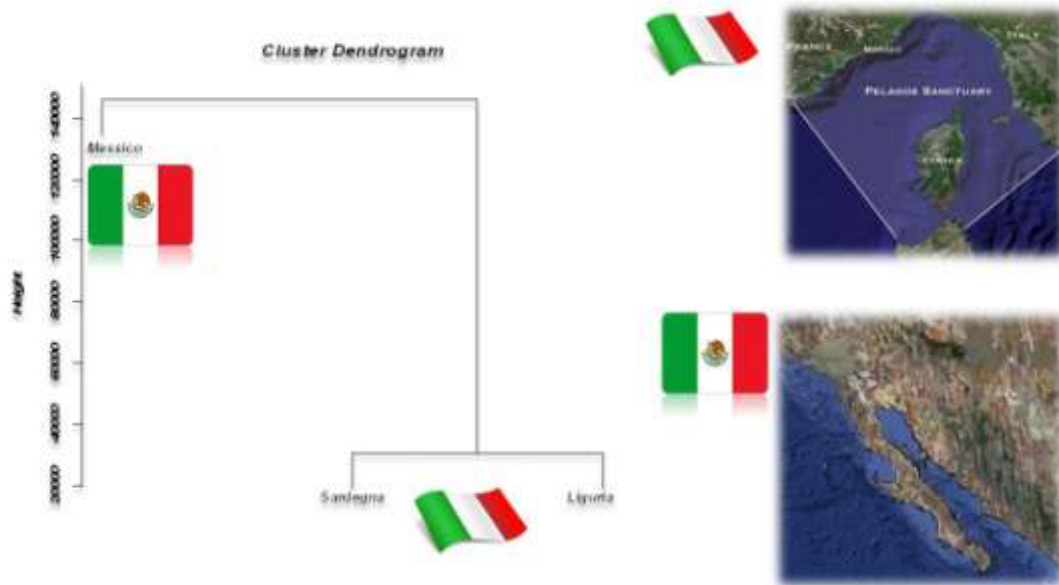


Fig.63. Livelli espressione dei geni PPAR G e PPAR A nei trattamenti ex-vivo in slices biopsia cutanea di un maschio di tursiope trattate con una miscela di ftalati (FT) a dosi crescenti.

4

CONSIDERAZIONI CONCLUSIVE



Considerazione conclusive

Vari aspetti vengono discussi nella parte conclusiva in funzione degli obiettivi progettuali:

- a) Principali considerazione sulle indagini ecotossicologiche su campioni di plancton/neuston e conteggio delle microplastiche nel Santuario Pelagos;
- b) Principali considerazione sulle indagini ecotossicologiche sul potenziale impatto delle microplastiche e suoi derivati tossicologicamente attivi su *B. physalus* e altre specie di cetacei del Santuario Pelagos;
- c) Principali considerazione sulle indagini ecotossicologiche sul potenziale impatto dei derivati delle microplastiche tossicologicamente attivi su *B. physalus* del Santuario Pelagos posti a confronto con esemplari campionati un area di “controllo” (Mar di Cortez-Messico).

A) Principali considerazione sulle indagini ecotossicologiche su campioni di plancton/neuston e conteggio delle microplastiche nel Santuario Pelagos

I risultati conclusivi di questo progetto sono fra i primi dati a livello internazionale sulla presenza di microplastiche nel Mar Mediterraneo e i possibili effetti sugli organismi. I dati prodotti riportano l'abbondanza e la distribuzione spaziale delle microplastiche nell'area del Santuario Pelagos in relazione a tre diverse aree di studio (Mar Ligure, Mar di Sardegna e Mar Tirreno) ed in funzione di 3 anni di campionamento (2011-2013).

In particolare nei 77 campioni superficiali di plancton/neuston campionati nei tre anni di progetto (2011-2013), il 75,3% conteneva particelle di microplastiche.

La media totale degli items/m³ presente nelle 77 retinate superficiali effettuate nelle tre aree del Santuario Pelagos è di 0,36 items/m³. Tra le diverse aree di campionamento risulta evidente come il valore medio dell'Arcipelago Toscano (0,80 items/m³) sia più alto rispetto alle altre due aree (Mar di Sardegna e Mar Ligure) prendendo in considerazione i diversi anni di campionamento.

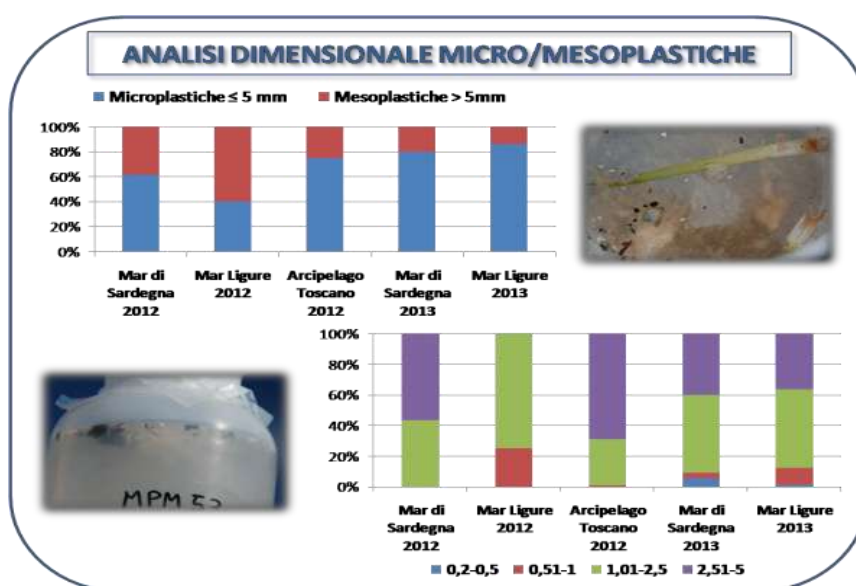


Fig.64. Valori % di delle varie categorie di microplastiche suddivise in base alle dimensioni nelle diverse aree ed anni di campionamento.

I dati di questo progetto rivelano inoltre che una delle aree più impattate da microplastiche del risulta l'AMP delle Cinque Terre. Questa area è stata confermata come *hot spot* per le microplastiche anche dai recenti studi di Collignon et al., 2012. Questi risultati sono importanti per focalizzare l'attenzione sullo stato di conservazione di un'area molto sfruttata dal turismo e sull'equilibrio tra misure di conservazione e di gestione.

Infine, da una accurata indagine dimensionale dei frammenti di plastica campionati nelle varie areedi studio, emerge che tutte le aree di campionamento presentano valori più alti di microplastiche (<5mm) rispetto alle mesoplastiche (>5mm). Inoltre, le categorie di microplastiche più presenti nei campioni superficiali (ad eccezione del campioni prelevati nel Mar Ligure nel 2012), siano quelle 1,01 - 2.5 ; 2,51 - 5 mm.

Infine, delle 11 retinate effettuate nella colonna d'acqua, nessuna presentava particelle di microplastica.

Tutti campioni di plancton/neuston superficiali e nella colonna d'acqua (ove possibile, in base alla quantità di materia organica prelevata) sono stati sottoposti ad analisi tossicologica del contenuto di ftalati evidenziando sia la presenza del principale additivo della plastica DEHP che del suo metabolita MEHP in gran parte dei campioni analizzati.

In conclusione va sottolineato che l'abbondanza media di microplastiche stimata in questo studio (Santuario Pelagos) è paragonabile a quella riscontrata in altre aree ad alto impatto antropico, rappresentando quindi il primo campanello d'allarme sul rischio emergente da microplastiche nell'unica AMP pelagica del Mar Mediterraneo. Le cause di questo elevato livello di contaminazione possono essere molteplici. Le plastiche, derivanti dal turismo costiero, la pesca ricreativa e commerciale, scarichi urbani, il traffico e l'industria marittima possono entrare direttamente nell'ecosistema marino e costituire un rischio per il biota sia come macroplastiche che, a seguito di processi di degradazione a lungo termine, come microplastiche.

In passato pochissimi studi hanno esplorato l'impatto delle microplastiche sugli organismi filtratori o altri animali planctofagi. Questo progetto rappresenta il primo studio al mondo che ha indagato sul potenziale impatto delle microplastiche su organismi filtratori di grande taglia, come le balenottere.

Nel Mar Mediterraneo, durante il recente *survey* di Collignon et al., 2014 è stata rivelata la presenza di microplastiche negli stomaci della specie ittica *Myctophum punctatum*. Inoltre, diversi studi riportano informazioni sulla ingestione di frammenti di plastica di diversa dimensione, colore e forma da parte di pesci epibentofagi ed iperbentofagi (*Ariidae*, *Scianidae*) di ambienti estuarini demersali nell'Atlantico sud ovest tropicale. Di recente è stata riportata la presenza di interazioni tra diverse specie di mammiferi marini e frammenti di plastiche marine così come l'ingestione di plastiche nella specie *Pontoporia blainvillei*. Ciononostante, gli effetti fisiologici e tossicologici della ingestione di plastiche da parte di organismi filtratori sono stati fino ad oggi poco indagati e poco compresi, così come le implicazioni della ingestione delle plastiche attraverso la rete alimentare.

B) Principali considerazione sulle indagini ecotossicologiche sul potenziale impatto delle microplastiche e suoi derivati tossicologicamente attivi su *B. physalus* del Santuario Pelagos

Da una approfondita indagine, il sistema geo-referenziato GIS, appare evidente come nelle due aree oggetto di studio del Santuario Pelagos, vi sia una sovrapposizione fra le aree di foraggiamento e relativo avvistamento/campionamento delle balenottere e veri e propri “Hot Spot” di presenza di microplastiche. Infatti il massimo addensamento di microplastiche in zone pelagiche, come già evidenziato anche in aree pelagiche remote del pianeta (North Pacific Gyre), appare sicuramente legato a correnti (a forma di Gyre) che causano l’addensamento di questi frammenti plastici in aree dove probabilmente sono anche presenti elevate concentrazioni di plancton e quindi più frequentate dalle balenottere per motivi di foraggiamento.

In queste are “hot spot” le balenottere risultano di conseguenza fortemente esposte all’assunzione delle microplastiche durante le elevate attività di filtrazione, e a seguito di questo, all’impatto tossicologico di contaminanti rilasciati dalle plastiche come gli additivi (ftalati), ma anche da composti liposolubili POPs ad esse adesi (OC, PAH, PBDEs).

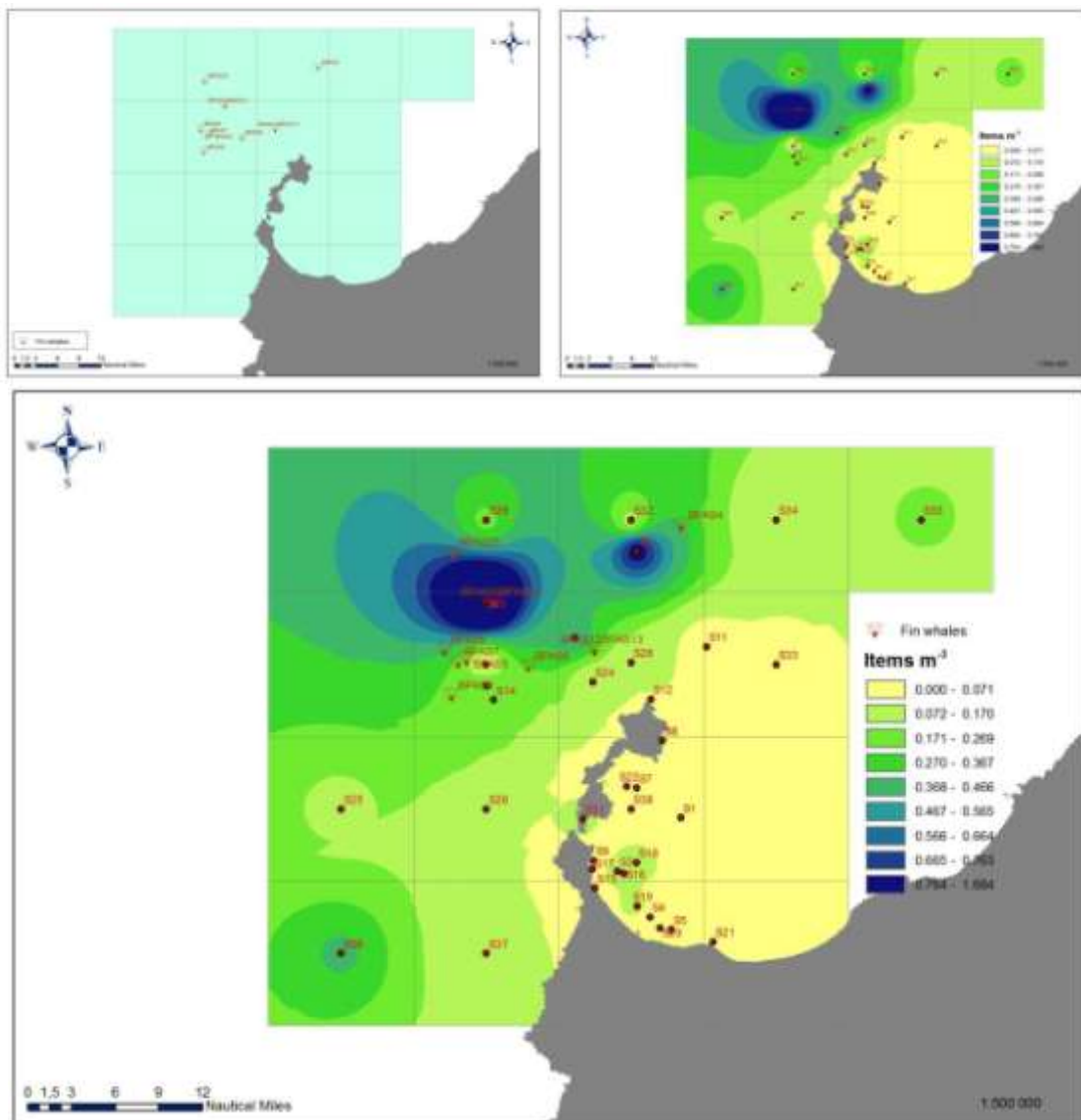


Fig.65. Distribuzione e densità delle microplastiche e punti di campionamento delle balenottere nell’area del Mar di Sardegna considerata.

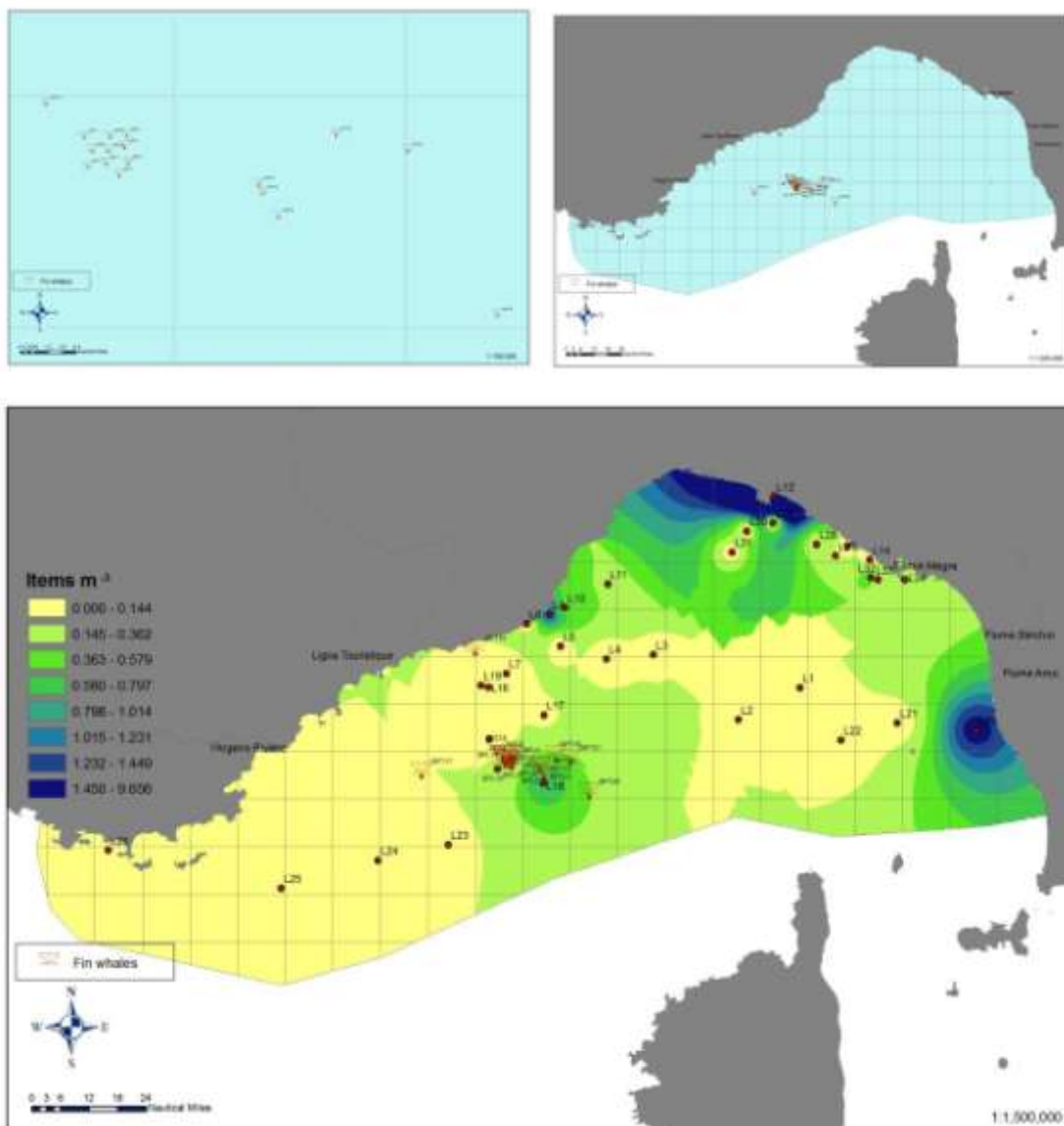


Fig.66. Distribuzione e densità delle microplastiche e punti di campionamento delle balenottere nell'area del Mar Ligure considerata.

Un calcolo teorico dell'assunzione di microplastiche nel Santuario Pelagos da parte di una balenottera comune è riportato nella tabella sottostante (Fossi et al 2014).

Tab.27. Calcolo dei parametri di alimentazione e ingestione di individui adulti di balenottera comune.

| | <i>Balaenoptera physalus</i> |
|--|------------------------------|
| Lunghezza media | 20 m |
| Peso corporeo | 50,000 kg |
| Volume di <i>engulfment</i> | 71 m ³ |
| Numero di <i>lunges</i> giorno⁻¹ | 83 |
| Volume totale filtrato al giorno | 5893 m ³ |
| Consumo totale di plancton al giorno | 913 kg |
| Numero teorico di microplastiche ingerite al giorno | 3653 |

Le plastiche e microplastiche in mare sono in grado di adsorbire e trasportare composti chimici, inclusi alti livelli di OC come PCB, DDT ed altri composti liposolubili come IPA. E' noto, inoltre, che additivi usati comunemente come i ritardanti di fiamma brominati, gli ftalati ed il monomero costituente bisfenolo A, possono agire come distruttori endocrini perché sono in grado di mimare, competere per o inibire la sintesi di ormoni endogeni. In particolare gli ftalati sono stati associati con vari effetti a livello molecolare, cellulare e di organo in invertebrati acquatici e pesci (Oehlmann et al., 2009). Il bisfenolo A è un agonista degli estrogeni ed antagonista degli androgeni e può alterare la riproduzione e lo sviluppo, in maniera differenziata a seconda delle concentrazioni in cui è presente e delle specie esposte. Dalle indagini eco tossicologiche effettuate sulle balenottere comuni che si alimentano nelle aree di studio emerge che le concentrazioni di OC indagate sono più elevate negli esemplari di sesso maschile campionati nel 2012, mentre per gli individui di sesso femminile il valore più elevato dei PCB è da attribuirsi ad i valori particolarmente elevati di un singolo individuo (BL10). La differenza nelle concentrazioni nei due anni oggetto di studio può essere attribuita al diverso periodo di campionamento, settembre (2012) e inizio agosto (2013) nel Mar Ligure e luglio nel Mar di Sardegna (2012 e 2013), e di conseguenza ad un maggiore bioaccumulo di composti liposolubili legato alla dieta negli individui campionati alla fine del periodo estivo di foraggiamento (Fig. 67). Nell'anno 2012 gli individui di balenottera comune, sia maschi che femmine, presentano dei livelli di espressione del CYP1A più elevati (le femmine di circa 10 volte) rispetto ai valori di espressione dello stesso gene campionati nella campagna del 2013. Questo dato è strettamente correlato alle diverse concentrazioni di contaminanti OC rilevate nei due anni/periodi di campionamento.

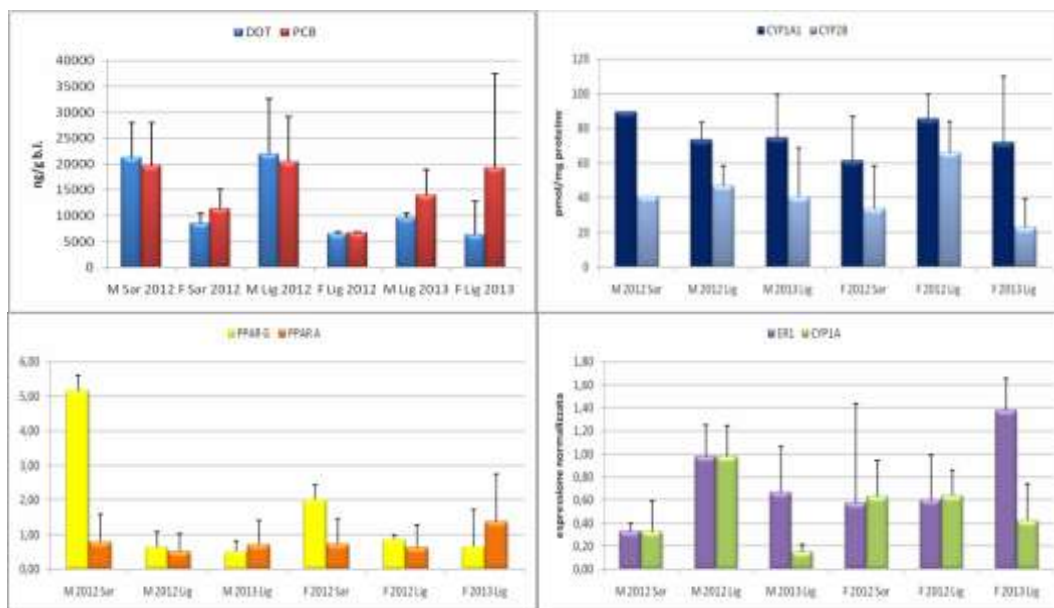


Fig.67a. Livelli di OC e risposte biomarkers suddivisi per sesso (M, F), anno di campionamento (2012, 2013), area di campionamento (Mar di Sardegna=Sar, Mar Ligure=Lig).

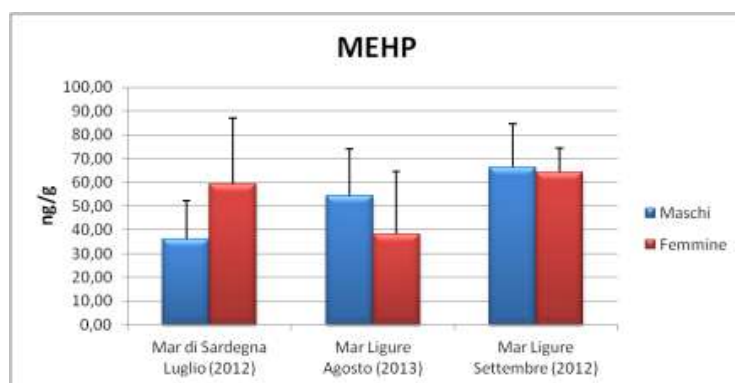


Fig.67B. Livelli ftalati suddivisi per sesso, anno e mese di campionamento e area di campionamento

C) Principali considerazione sulle indagini ecotossicologiche confrontando *B. physalus* del Santuario Pelagos con esemplari campionati un area di “controllo” (Mar di Cortez-Messico).

Allo scopo di valutare due aree a diverso impatto antropico è stata effettuata l'analisi su campioni di *B. physalus* campionate sia nel Santuario Pelagos che nell'area “di controllo” del Mar di Cortez (Mexico). I dati presi in esame si riferiscono sia ai livelli di contaminanti e alle risposte biomarker (Riportati nel Report del 1 anno di attività).

Da questa indagine comparativa emerge che le balenottere del Santuario Pelagos hanno una concentrazione di MEHP superiore rispetto all' area del Mar di Cortez, infatti hanno una concentrazione media di MEHP pari a 59,54 ng/g peso secco, mentre in quelli del Mar di Cortez la concentrazione è di 39,98 ng/g peso secco. Anche dalle analisi degli organoclorurati totali (DDT totali e i PCB totali) emerge che le concentrazioni negli individui provenienti dal Santuario Pelagos risultano più alte rispetto a gli individui campionati nel Mar di Cortez (eccetto per l'HCB).

Le analisi delle risposte dei biomarker quali il citocromo P450 1A1/2B mediante western blot, la perossidazione lipidica e l'espressione genica del recettore per gli estrogeni alfa mostrano in tutti i test diagnostici utilizzati una risposta più alta negli individui del Santuario Pelagos rispetto a quelli del Mar di Cortez (eccetto CYP2B, maggiormente legato a processi di detossificazione), confermando il più alto rischio tossicologico dell'area Mediterranea rispetto all'area Messicana, in particolare in relazione alla presenza di composti con potenzialità da distruttori endocrini.

La balenottera come indicatore dello stato ecotossicologico di due bacini: mar Mediterraneo e Mar di Cortez

Al fine di realizzare un'analisi conclusiva dei dati e di proporre la balenottera comune come *indicatore integrato della contaminazione di un intero bacino*, sono stati analizzati i dati relativi alle balene campionate nel Mar Mediterraneo (Santuario Pelagos) ed in Mar di Cortez (Messico) con le seguenti tecniche di analisi statistiche:

- A) Analisi gerarchica dei clusters: applicata allo scopo di verificare l'esistenza di sub-aree omogenee all'interno dell'area investigata. Il numero dei clusters ottimo è stato determinato applicando la regola del “Cut”.
- B) Analisi discriminante sui fattori della Principal Component Analysis: applicata allo scopo di evidenziare i parametri discriminanti i clusters definiti al punto A). La significatività dell'analisi è stata testata attraverso il Monte-Carlo Test della somma degli autovalori (divisi attraverso i ranghi) di una analisi discriminante. Versione non parametrica del Pillai test.

Tutte le analisi sono state svolte utilizzando il software R version 2.15.2 (2012-10-26), "Trick or Treat" ed i packages Energy, Ade4.

Dall'analisi dei cluster si evince la presenza di due principali raggruppamenti distinti che raccolgono le osservazioni (considerando tutte le variabili) nel Mar Mediterraneo (Santuario Pelagos, Mar di Sardegna e Mar Ligure) e nel Mar di Cortez (Fig. 64).

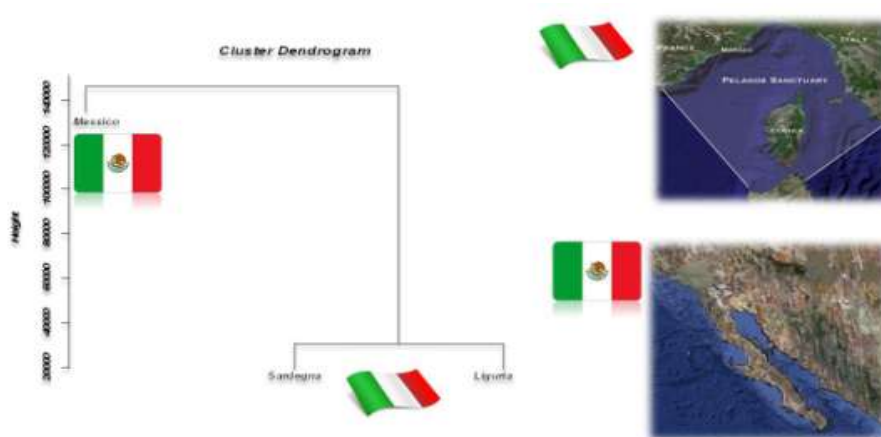


Fig. 68. Dendrogramma dell'analisi gerarchica dei clusters.

Sui gruppi di osservazione è stata quindi applicata l'analisi discriminante sui fattori della PCA allo scopo di evidenziare le variabili cui può essere imputata una eventuale discriminazione delle aree esaminate. Tale analisi è stata svolta considerando le osservazioni sugli individui di balenottera del Mar di Cortez e delle due aree del Santuario Pelagos)

Le figure 69 e 70 mostrano i risultati dell'analisi, prima separando le tre popolazioni di dati e successivamente solo comprendo gli esemplari provenienti dai due bacini.

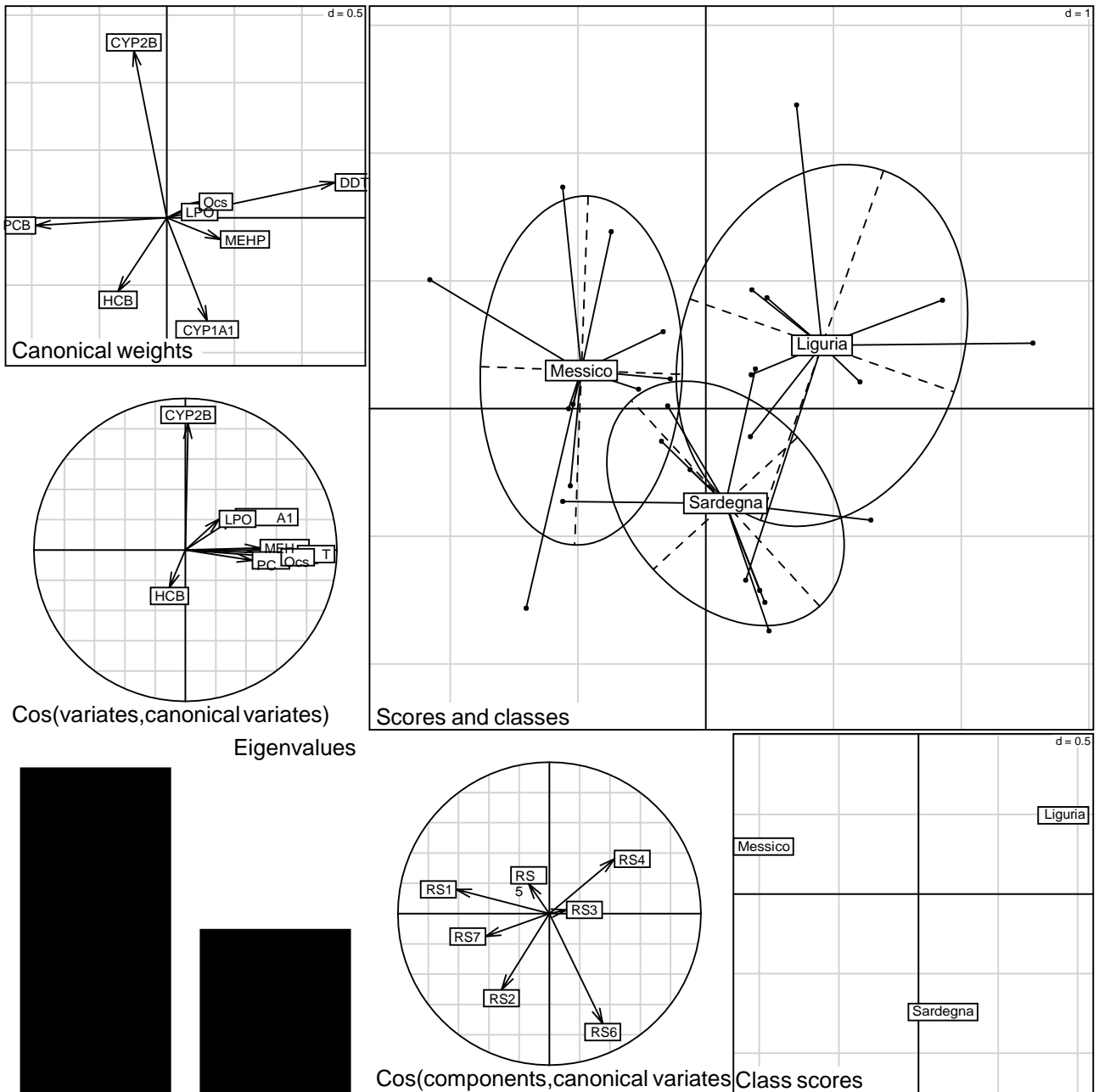


Fig. 69. Analisi discriminante sui fattori della PCA. Grafico composto: Plot dei pesi canonici (in alto a sinistra); rappresentazione della matrice di struttura: plot delle correlazioni fra le variabili discriminanti e gli assi discriminanti (al centro a sinistra); Istogramma degli autovalori (in basso a sinistra); plot delle correlazioni fra i fattori della PCA e gli assi discriminanti (al centro in basso); plot dei centroidi di gruppo (in basso a destra); plot dei punteggi canonici (main plot).

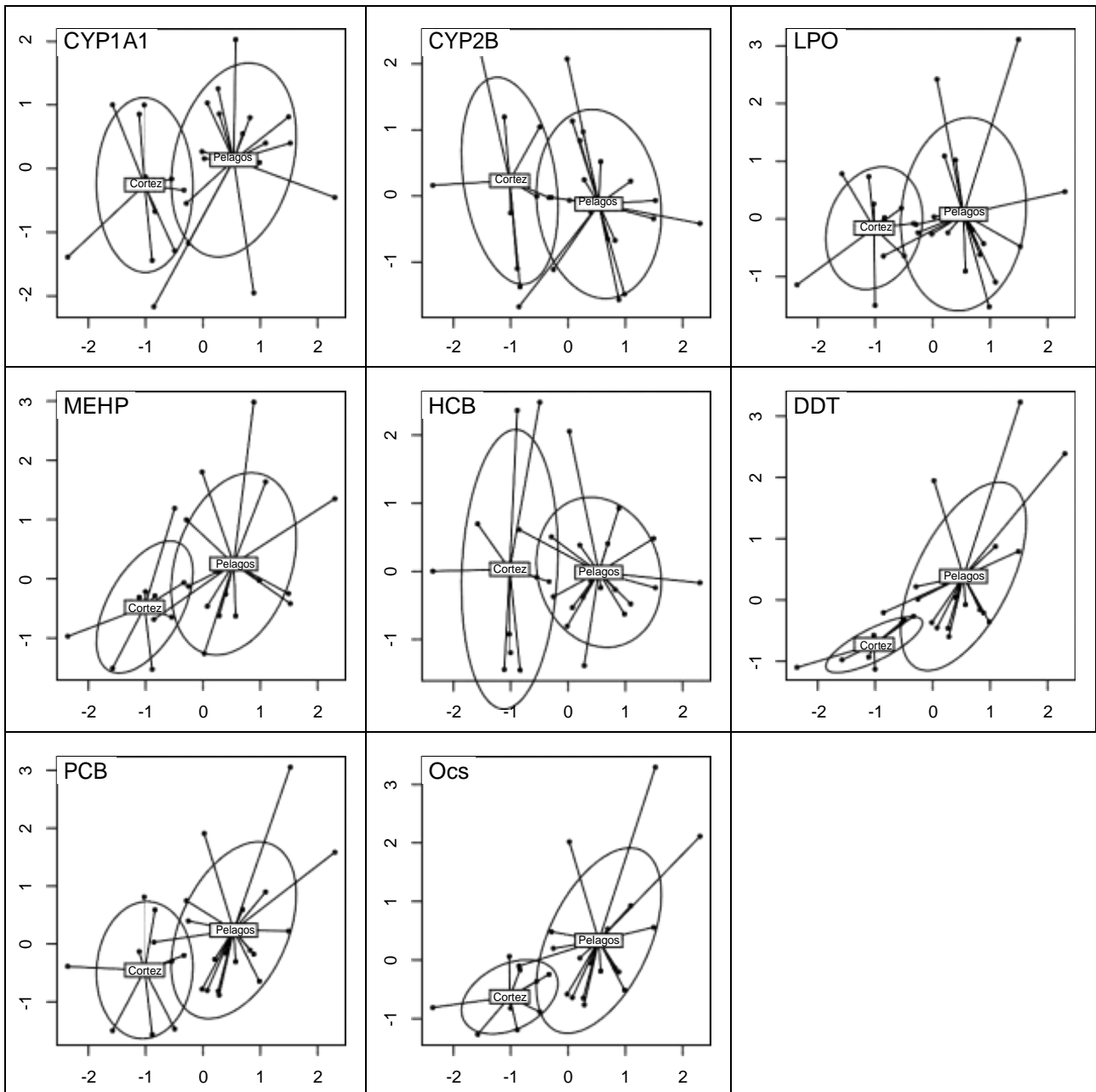


Fig. 70. Analisi discriminante sui fattori della PCA. Grafico dei caninical scores by discriminant variables

Il grafico, diverso dal precedente perché, in presenza di due soli gruppi viene definito un solo asse discriminante, rappresenta, per ciascuna variabili discriminante, i canonical scores. Dalla posizione che i centroidi di gruppo assumono lungo l'asse $y=0$ si osserva:

- 1) Le variabili HCB e LPO non sembrano discriminare i due bacini
- 2) Nell'ordine le variabili DDT, OC, MEHP, PCB discriminano i due bacini essendo prevalenti in Pelagos
- 3) CYP1A1 e CY2B hanno un effetto discriminativo più blando: la prima assume valori più elevati in Pelagos, la seconda valori più elevati in Cortez.

Il test di Montecarlo replicato 999 volte è risultato significativo (RV=0.08 $p=0.006$) a riprova del fatto che il modello discriminante applicato è significativo.

In conclusione I dati ottenuti in questo progetto (primo e secondo anno di attività) rappresentano la prima evidenza a livello mondiale del potenziale impatto dei più abbondanti derivati delle plastiche (gli ftalati) nel secondo più grande mammifero planctofago, la balenottera comune. Questi dati sottolineano inoltre l'elevata presenza di microplastiche nelle varie aree oggetto di studio del Santuario Pelagos, sottolineando il rischio di esposizione della cetofauna che frequenta la suddetta area.

Questi dati, presentati sia in lavori scientifici pubblicati su importanti riviste del settore che in vari contesti congressuali internazionali (vedi allegato 2) hanno avuto un particolare riconoscimento sullo scenario internazionale, soprattutto da parte dell' IWC come confermato da: a) comitato scientifico dell' International Whaling Commission - Panama 2012, b) Marine debris workshop IWC – Wood Hole 2013; c) Steering Committee di Pollution 2020 (St. Andrews 2014), dove uno dei temi prioritari è l'impatto delle plastiche e microplastiche sui cetacei, con particolare riferimento al caso di studio della balenottera comune del Mediterraneo e al presente progetto.

La balenottera comune è un cetaceo cosmopolita. E' presente nelle più ampie masse d'acqua del pianeta, dall'equatore alle regioni polari. Nonostante la sua distribuzione cosmopolita è classificata come *endangered* nella lista rossa dell'IUCN. In generale, l'alimentazione delle balenottere è stata descritta come l'evento biomeccanico più grande mai esistito sulla terra. La balenottera si nutre all'inizio nuotando rapidamente verso le prede e poi decelerando mentre apre la bocca per ingerire enormi quantità di acqua e prede. La balenottera comune e la balenottera azzurra che si nutrono di krill concentrano i loro sforzi su grandi aggregazioni di krill (150-300 m) nella colonna d'acqua durante il giorno e si nutrono in superficie durante la notte.

E' ben noto che la balenottera nel Mediterraneo si nutre di preferenza dell'eufasiaceo planctonico *Meganyctifanes norvegica*, ciò nonostante, a seconda dell'area e della stagione, si nutre anche di un ampio spettro di organismi marini, inclusi copepodi, altre specie di eufasiacei e piccoli pesci. Ad ogni movimento di ingestione una balenottera può assumere circa 70.000 l di acqua. Per questo motivo è a rischio per l'ingestione di una quantità molto elevata di frammenti di plastica sia direttamente dall'acqua che indirettamente dal plancton (sia durante l'alimentazione superficiale che durante quella profonda). Dopo l'ingestione delle microplastiche la balenottera può essere esposta al rilascio di additivi come OC, PBDE, ftalati ed bisfenolo A ed ai loro potenziali effetti tossicologici.

Dati preliminari sul MEHP in 5 campioni di eufasiacei quali *Euphausia krohni*, campionati nel canale di Sicilia, riportano alti livelli di questo contaminante, con range tra 8,35 e 51,14 ng/g. Questi risultati suggeriscono la presenza di derivati delle plastiche anche in specie planctoniche residenti nella colonna d'acqua.

Considerando la presenza di microplastiche nell'ambiente Mediterraneo, la presenza di additivi delle plastiche nel grasso sottocutaneo di esemplari di balenottera (sia spiaggiati che free-ranging) e la lunga aspettativa di vita di questa specie, la balenottera appare esposta in maniera cronica ed elevata a contaminanti emergenti persistenti come risultato della ingestione delle microplastiche. In questo stesso contesto, i risultati ottenuti suggeriscono che gli ftalati possono essere usati come traccianti per l'assunzione delle microplastiche da parte della balenottera sia direttamente dalla filtrazione del mezzo acquatico che dall'ingestione di plancton già contaminato dagli stessi additivi della plastica.

Questi dati rappresentano un primo segnale di allarme sul rischio potenziale che la popolazione mediterranea di balenottera comune sia esposta a distruttori endocrini come il MEHP. Questi risultati sono in linea con quanto riportato in precedenza da Fossi et al., 2010, riguardo al pericolo da distruttori endocrini in questa specie nel mediterraneo. I "*undesirable biological effects*" (in

accordo con il concetto di biomarker presente nel Descrittore 8/10 della Strategia Marina Europea) evidenziati suggeriscono come la popolazione mediterranea di balenottere possa essere esposta ad una miscela di contaminanti persistenti ed emergenti tra cui gli interferenti endocrini, che possono alterare la sopravvivenza di questa popolazione già considerata *endangered*.

A questo riguardo, censimenti condotti in gran parte del Mediterraneo occidentale hanno stimato che la popolazione della balenottera fosse di 3.583 individui, di cui 901 localizzati nel bacino corso-ligure-provenzale (Forcada 1995 e 1996). Dati più recenti riguardanti il Santuario Pelagos indicano che la popolazione stimata è diminuita marcatamente (approssimativamente di un fattore 6) negli ultimi 20 anni (Panigada et al, 2011), focalizzando l'attenzione sulle problematiche di conservazione di questa specie.

In conclusione, i presenti dati hanno rappresentato la prima evidenza a livello mondiale del potenziale impatto delle microplastiche e derivati (additivi delle plastiche-ftalati) nei cetacei misticeti ed in particolare nella balenottera comune. Questi risultati sottolineano l'importanza di future ricerche riguardo lo studio dell'impatto tossicologico delle microplastiche in specie filtratrici come i cetacei misticeti, lo squalo elefante (*Cetorhinus maximus*) ed la mobula (*Mobula mobula*) (Fossi et al. 2014)

Questi risultati rafforzano inoltre l'uso potenziale di queste specie per l'implementazione del Descrittore 10 (marine litter) della Direttiva Quadro della Strategia Marina Europea come indicatori della presenza e dell'impatto di microplastiche nell'ambiente pelagico (Fossi et al. 2014, Galgani et al. 2014).

PRINCIPALI RISULTATI OTTENUTI DAL PROGETTO E POSSIBILI SVILUPPI FUTURI

Questi risultati rappresentano la prima evidenza a livello mondiale del potenziale impatto delle microplastiche e derivati (additivi delle plastiche-ftalati) nei cetacei misticeti ed in particolare nella balenottera comune. Questi importanti risultati conseguiti hanno ottenuto un riconoscimento sullo scenario internazionale come confermato dal comitato scientifico dell'International Whaling Commission (Panama 2012, Woods Hole 2013, St. Andrews 2014) e come confermato dalla necessità di implementare il Descrittore 10 (Marine Litter) della Direttiva Quadro della Strategia Marina Europea con lo studio di grandi organismi filtratori (Fossi et al. 2014, Galgani et al. 2014).

In sintesi i principali risultati del progetto in funzione degli obiettivi proposti sono:

- E' stato realizzato un protocollo metodologico standard di riferimento per l'analisi di microplastiche e derivati tossicologicamente attivi (ftalati) in campioni di plancton e micro-layer superficiale (incorporato nell'attuale Monitoraggio per il Descrittore 10 da parte delle Regioni)
- E' stata effettuata una mappatura delle microplastiche in un area più vasta del Santuario Pelagos comprendente le aree del Mar Ligure, Mar di Sardegna e Mar Tirreno (Arcipelago Toscano). E' stato inoltre realizzato un primo conteggio e mappatura delle Macroplastiche superficiali nelle varie aree di indagine del Santuario Pelagos
- E' stato realizzato il primo protocollo metodologico standard di riferimento per l'analisi di composti potenzialmente tossici derivati da microplastiche in campioni di biopsie cutanee di balenottera comune e di altre specie di cetacei residenti nel Santuario Pelagos.
- Sono state sviluppate metodologie diagnostiche *ex vivo* (*slices* di biopsie cutanee) per evidenziare l'esposizione e l'effetto dei composti derivati da microplastiche o da loro veicolati, al fine di diagnosticare gli effetti biopsie cutanee di individui *free-ranging*.
- E' stata realizzata una prima valutazione dei livelli e degli effetti dei composti derivati da microplastiche, o da loro veicolati, sulla popolazione di balenottera comune campionata nell'area del Santuario Pelagos e paragonata con un'area a ridotto impatto antropico (Mar di Cortez – Messico). Le stesse indagini sono inoltre state realizzate su individui *free-ranging* di altre specie di cetacei: stenella striata, delfino comune, grampo e tursiope.
- Sono state sviluppate tecniche diagnostiche più specifiche per evidenziare la presenza di microplastiche in individui planctonici e neustonici (rilevamento con tecniche di microscopia ottica e microscopia elettronica a scansione e trasmissione);
- E' stata proposta la prima bozza di applicabilità del "modello balenottera" come indicatore della qualità di "mega-aree" pelagiche (vedi Risultati Fase III, IV). L'importanza di questo risultato è connesso al conseguimento per la prima volta informazioni per determinare la presenza o meno di effetti ecotossicologici diretti connessi alla presenza di rifiuti galleggianti e loro derivati sugli ecosistemi marini, determinazione che risulta di estrema importanza per l'acquisizione di dati ed informazioni finalizzate al popolamento del descrittore 10 (*marine litter*) per l'implementazione della direttiva quadro sulla strategia europea dell'ambiente marino, per il quale non esistono al

momento metodiche alternative.

A seguito degli importanti risultati conseguiti dallo svolgimento del presente si ritiene di particolare importanza il completamento e pianificazione di attività progettuali future sui seguenti aspetti:

- Completare la mappatura delle microplastiche nell'intero Santuario Pelagos monitorando le aree non indagate in questa fase progettuale.
- Espandere l'area di indagine, sia per il mappaggio delle microplastiche che per le indagini ecotossicologiche sulla Balenottera comune nei quartieri invernali del Canale di Sicilia (area di Lampedusa).
- Approfondire gli aspetti tossicologici legati alla presenza di altri composti tossici rilasciati o trasportati dalle plastiche (Ritardanti di fiamma, Bisfenolo A, ecc).
- Questi risultati sottolineano inoltre l'importanza di future ricerche riguardo lo studio dell'impatto tossicologico delle microplastiche in altre specie filtratrici come lo squalo elefante (*Cetorhinus maximus*) ed la mobula (*Mobula mobula*) (Fossi et al. 2014).

COLLABORAZIONI

Durante lo svolgimento del progetto, l'Università di Siena ha collaborato con i seguenti ricercatori ed istituti di ricerca.

Per le campagne di campionamento:

Dr. Giancarlo Lauriano, ISPRA, Roma

Dr. Simone Panigada, Tethys Research Institute, Milano

Dr. Jorge Urban, UBCS, Messico

Per la analisi di laboratorio e dei dati:

Dr. Maria Grazia Finoia, ISPRA, Roma

Prof. Pietro Lupetti, Università di Siena

Dr. Fabrizio Rubegni, Regione Toscana

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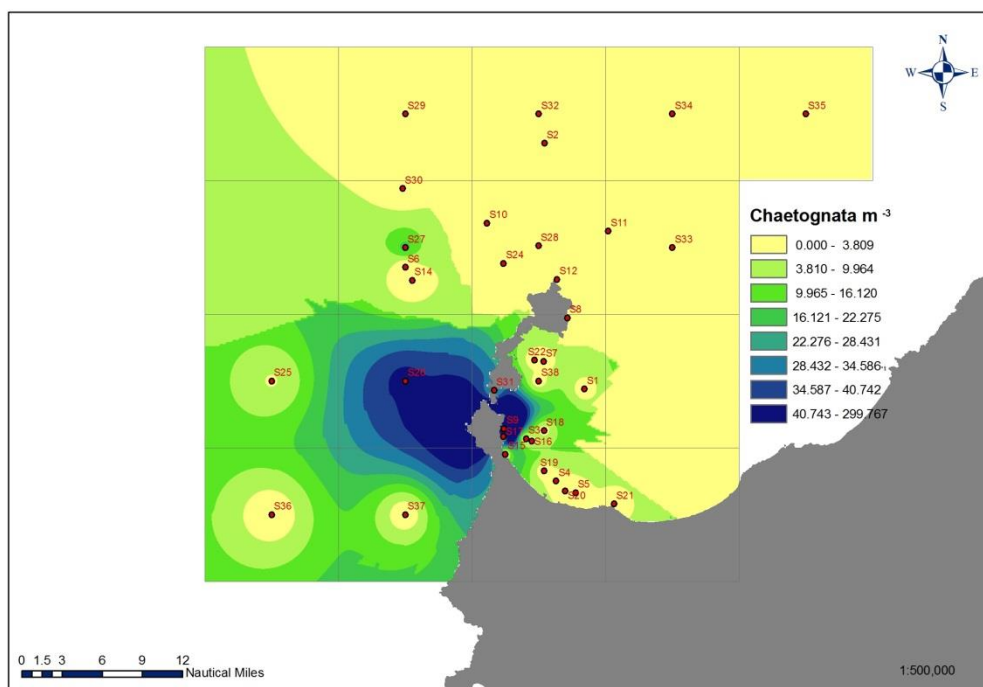
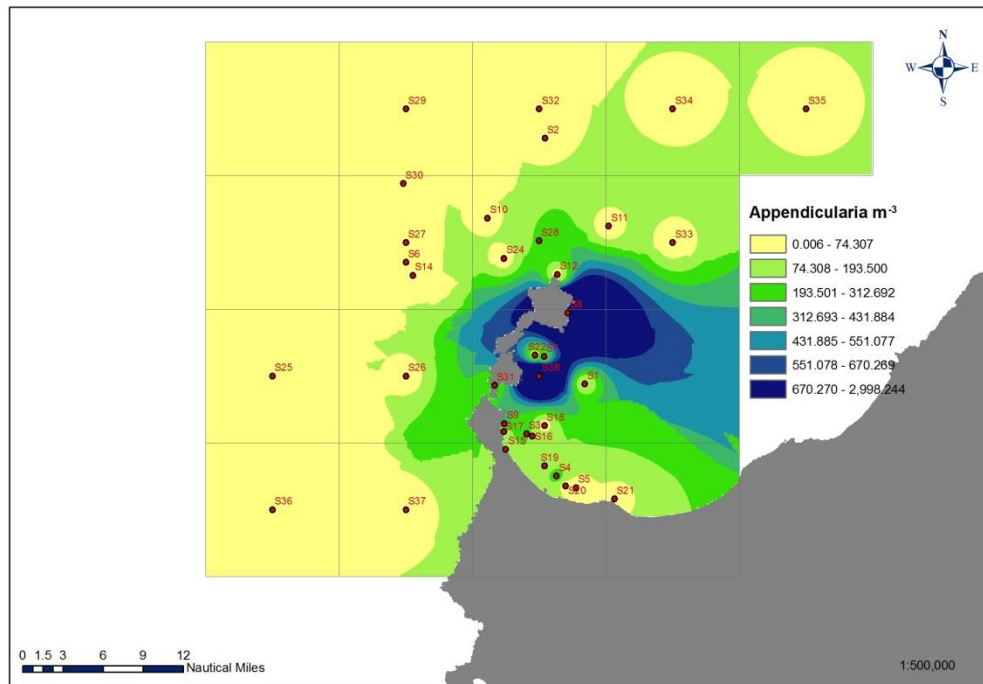
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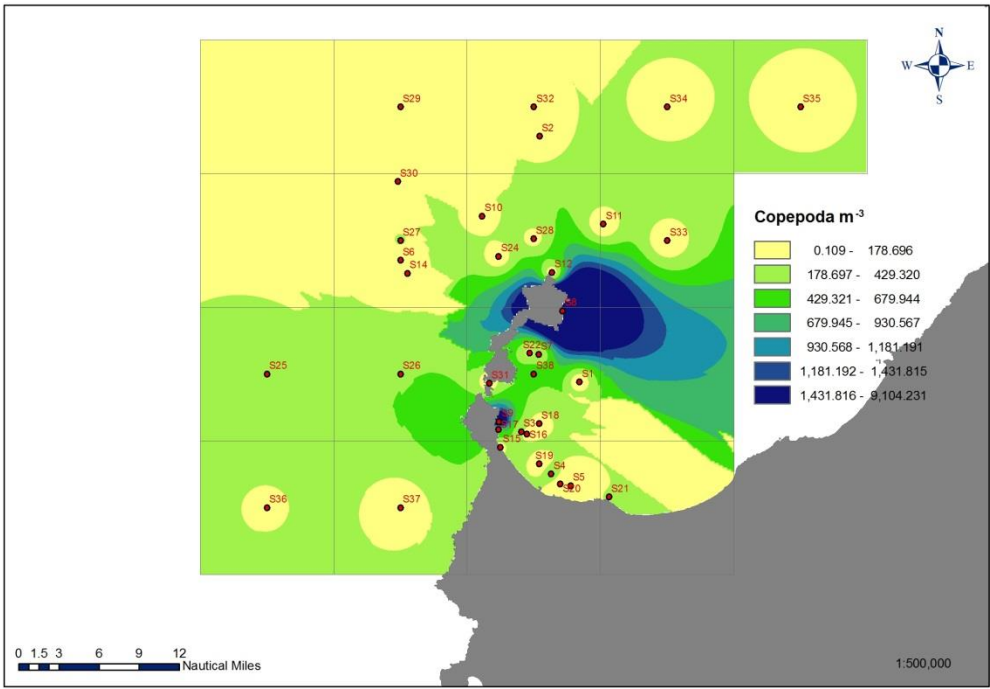
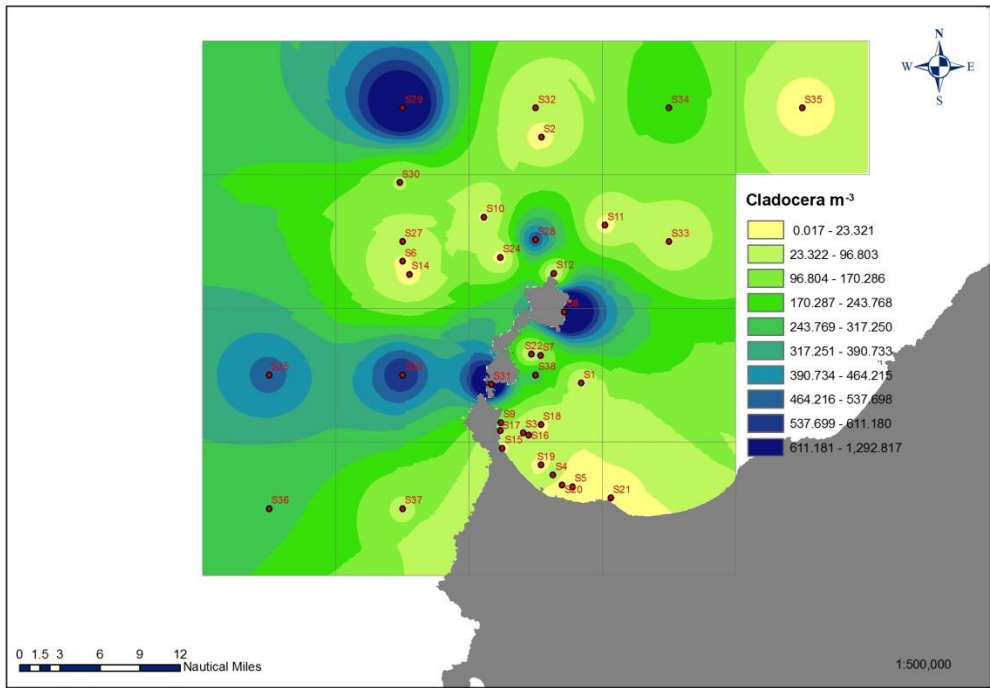
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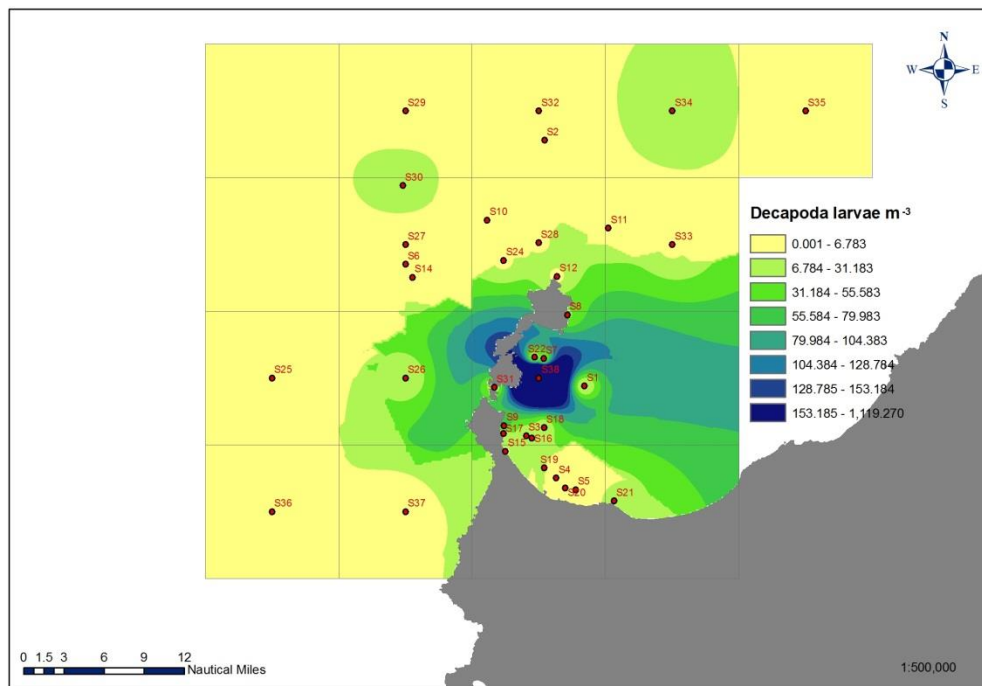
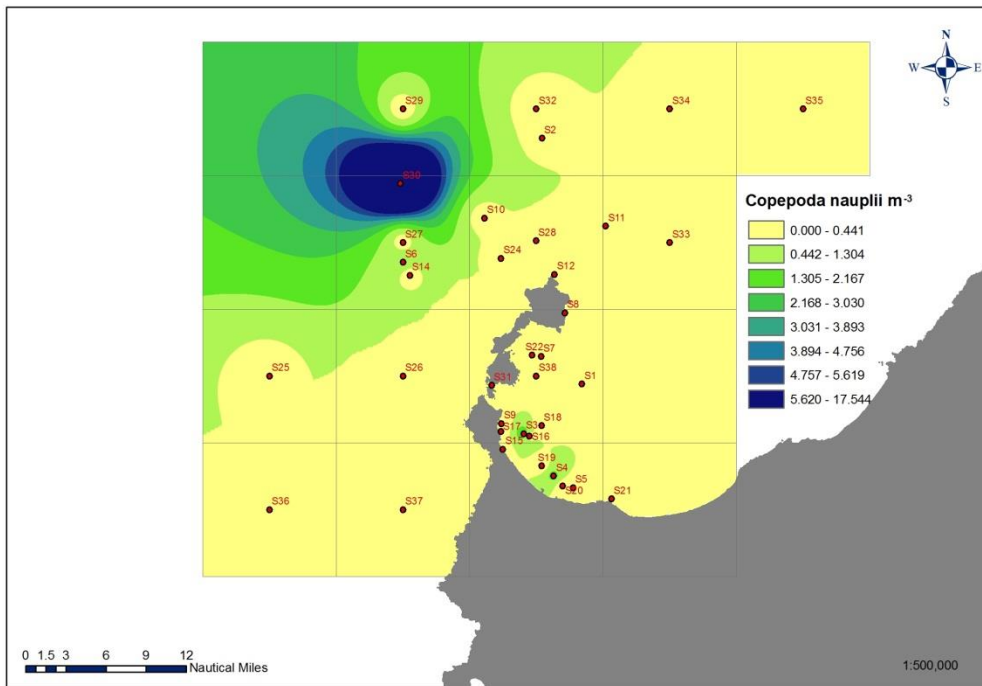
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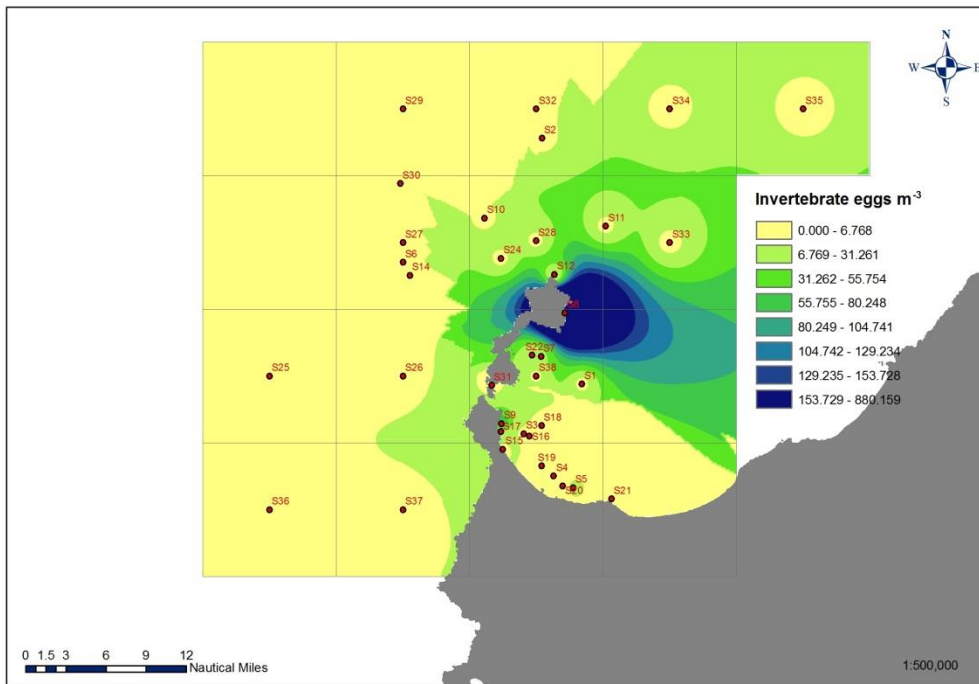
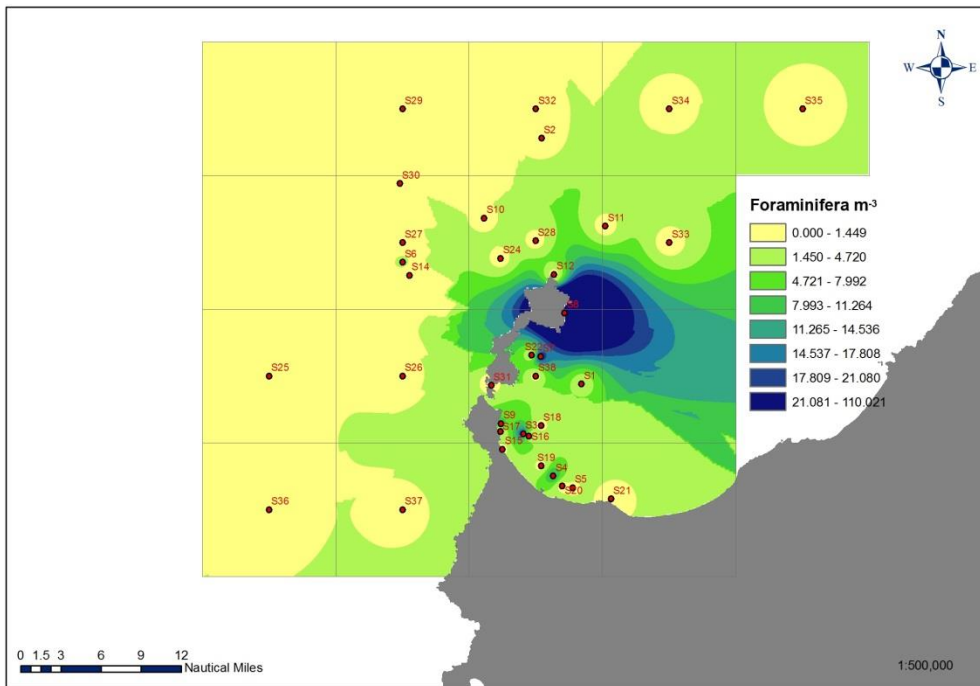
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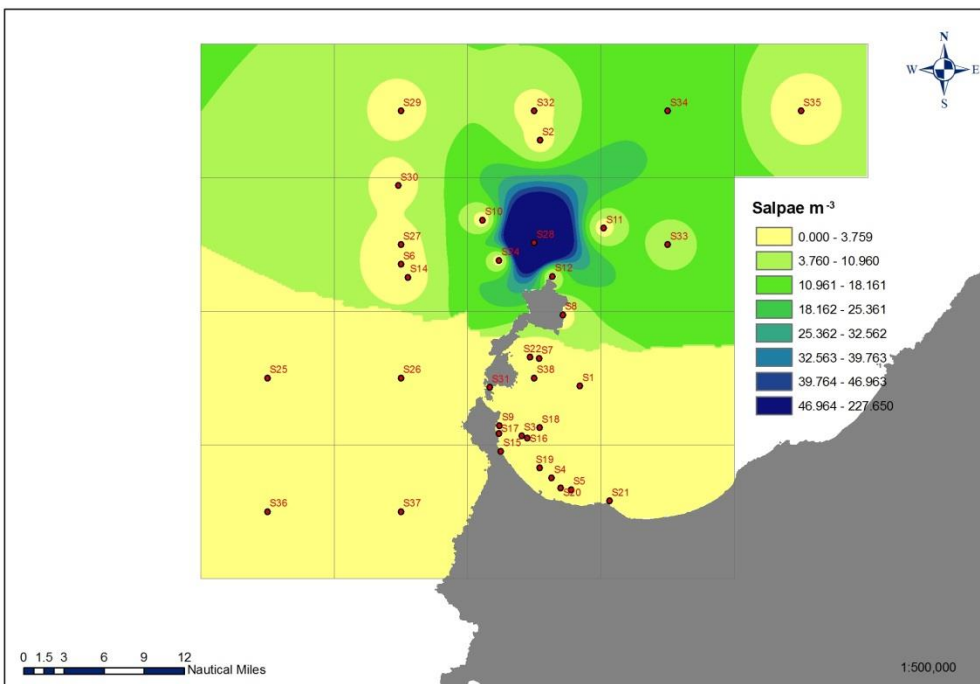
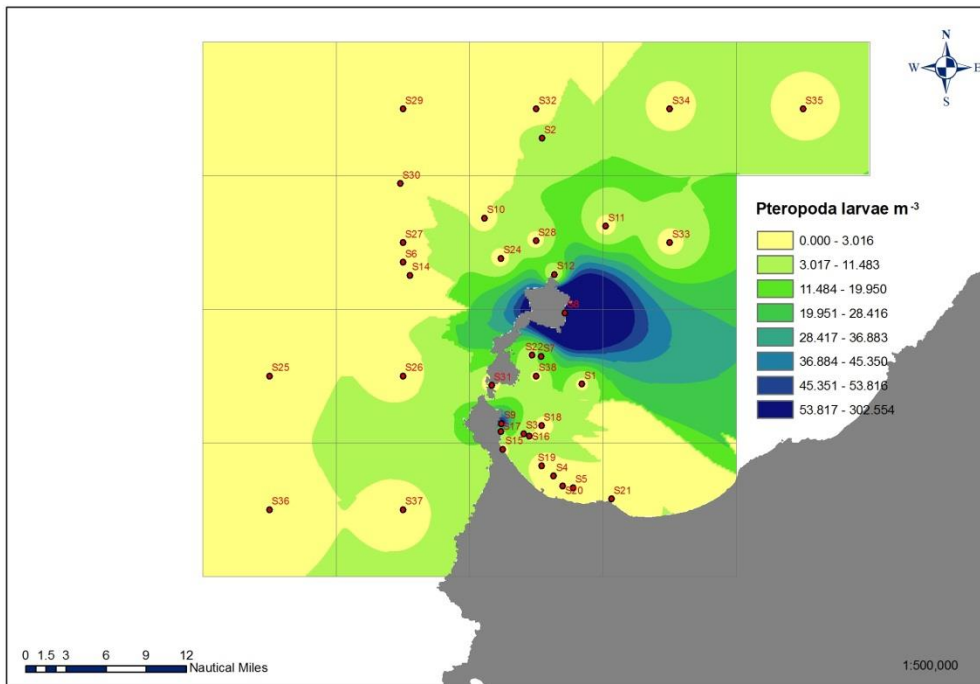
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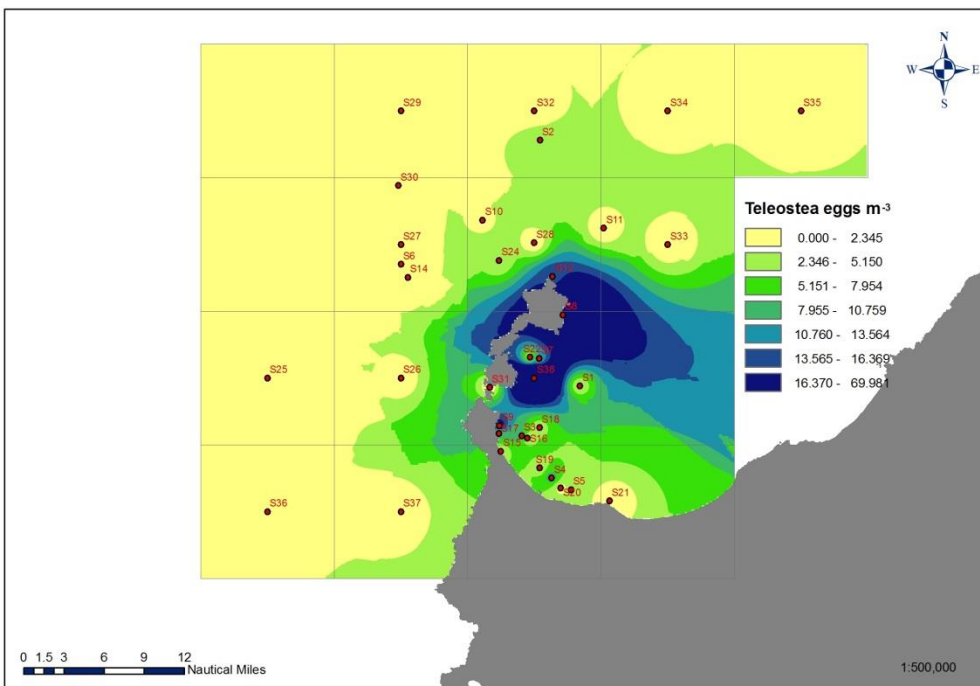
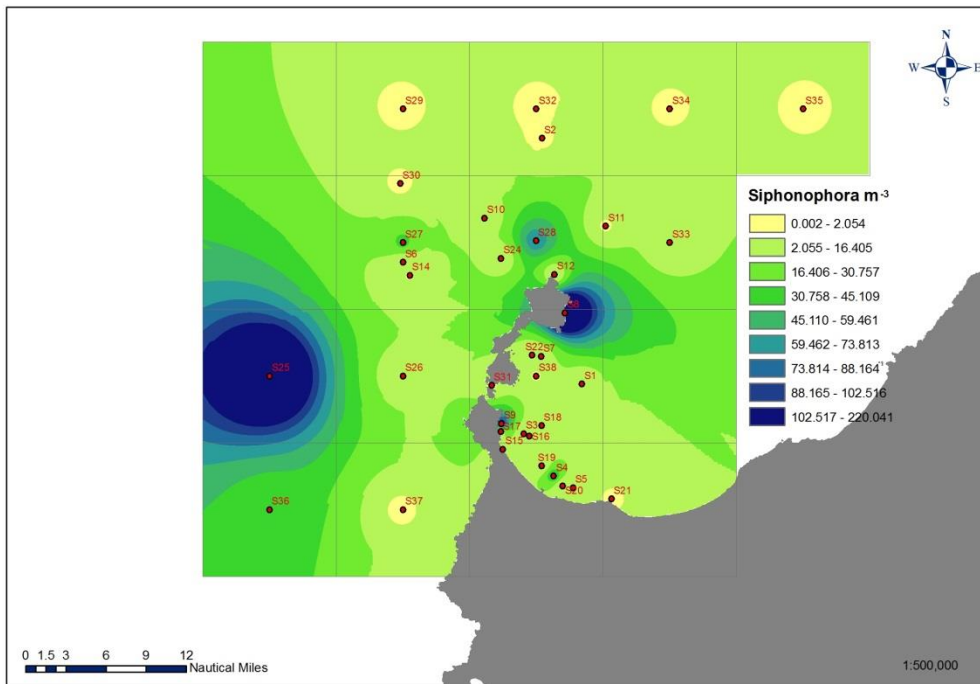






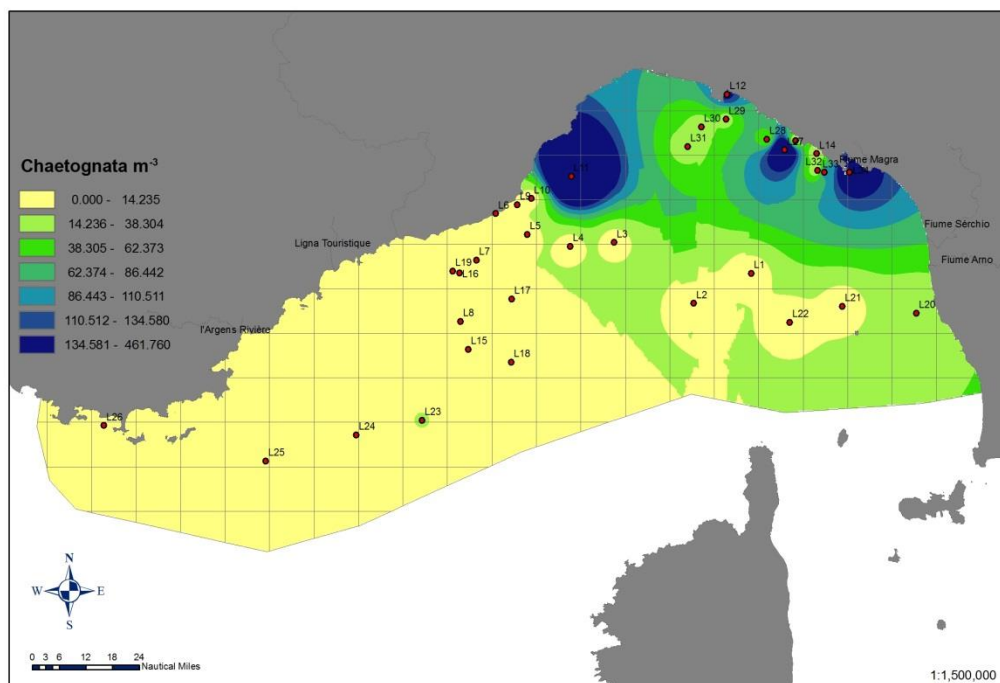
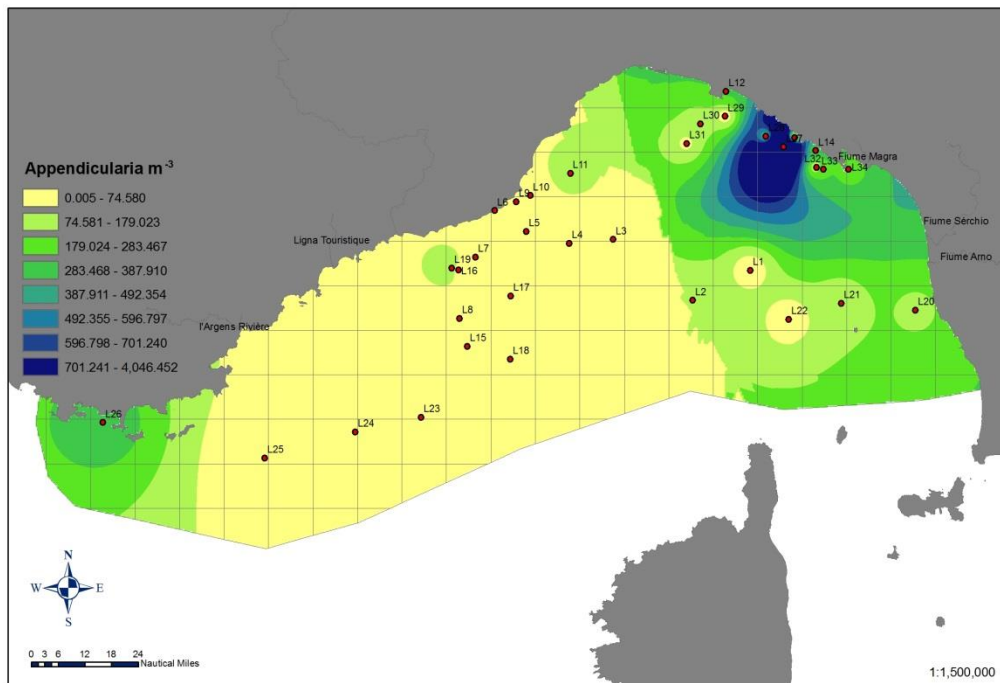


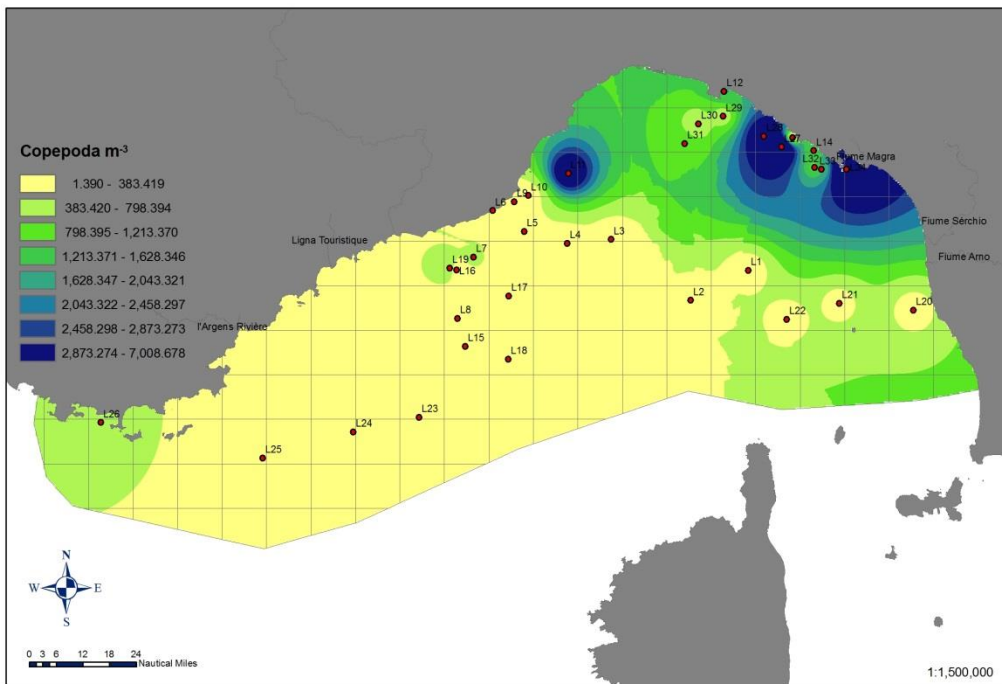
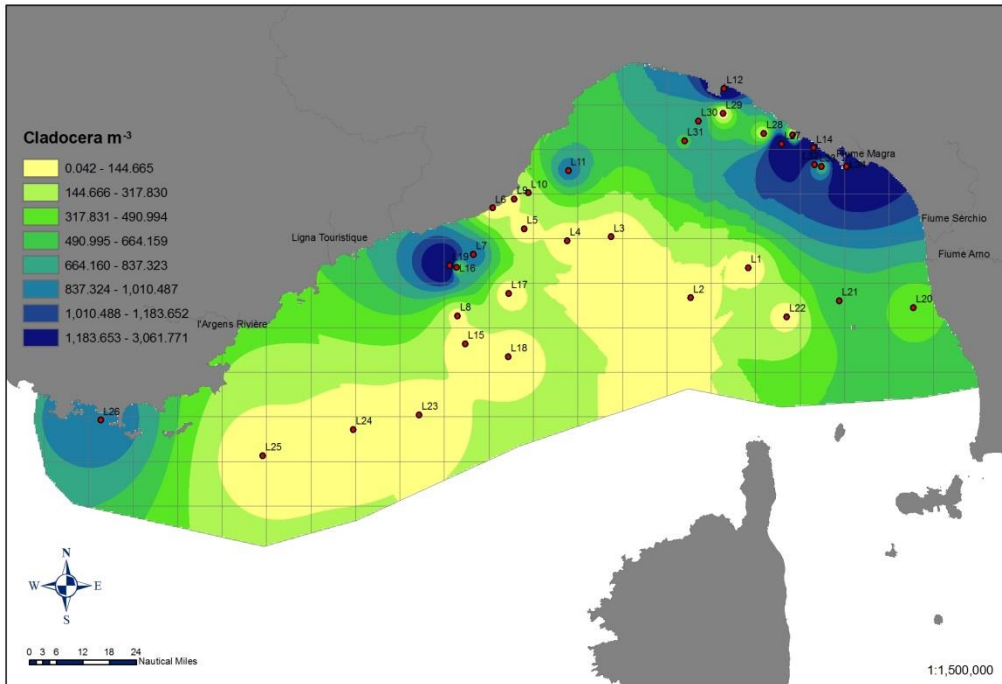


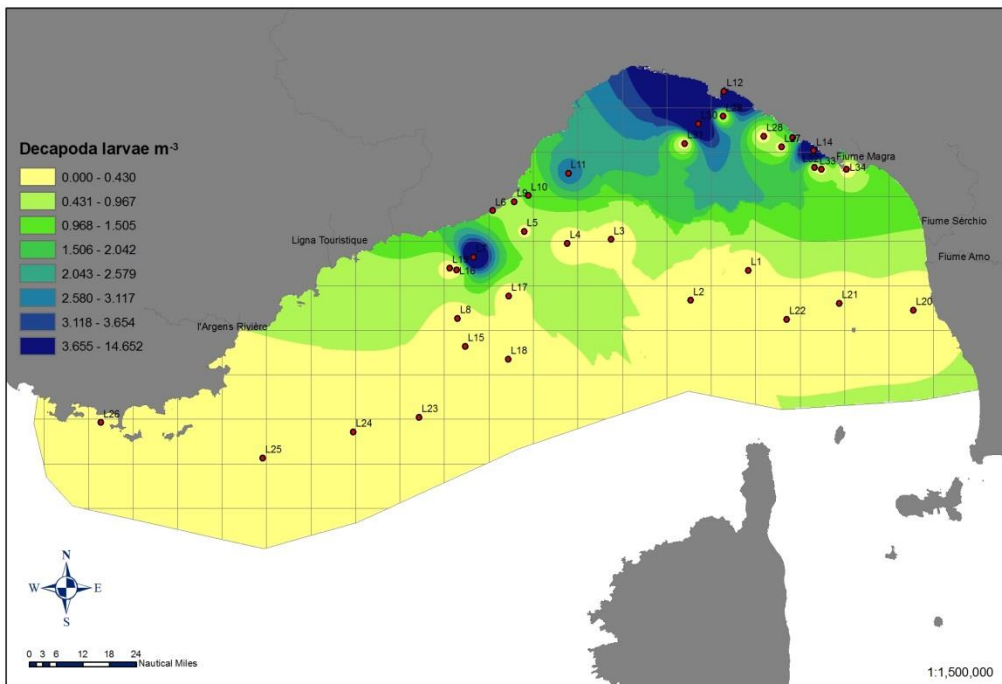
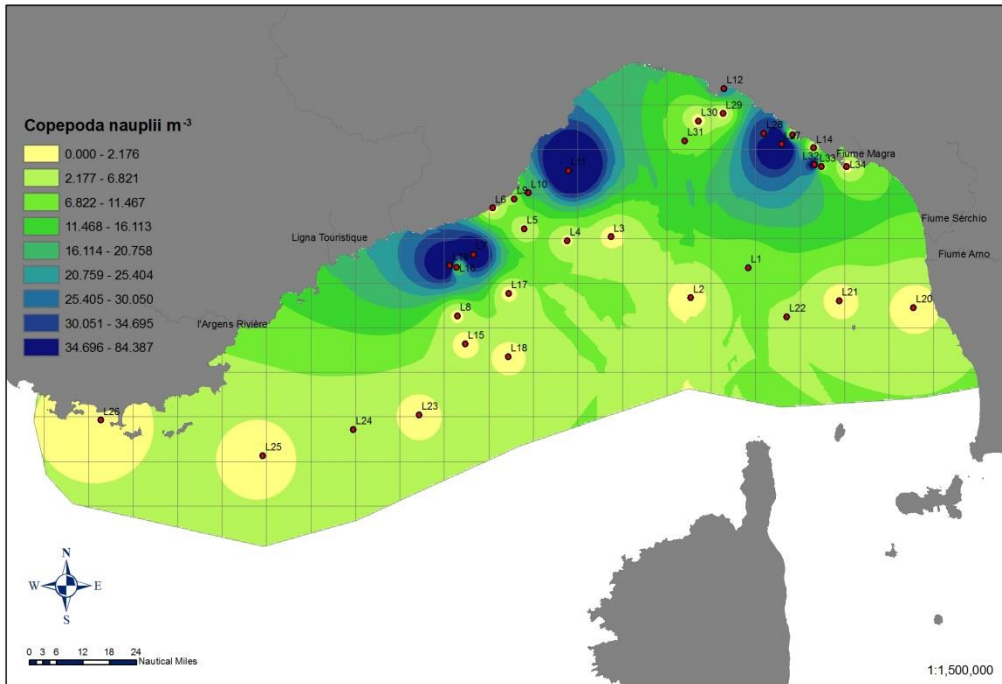


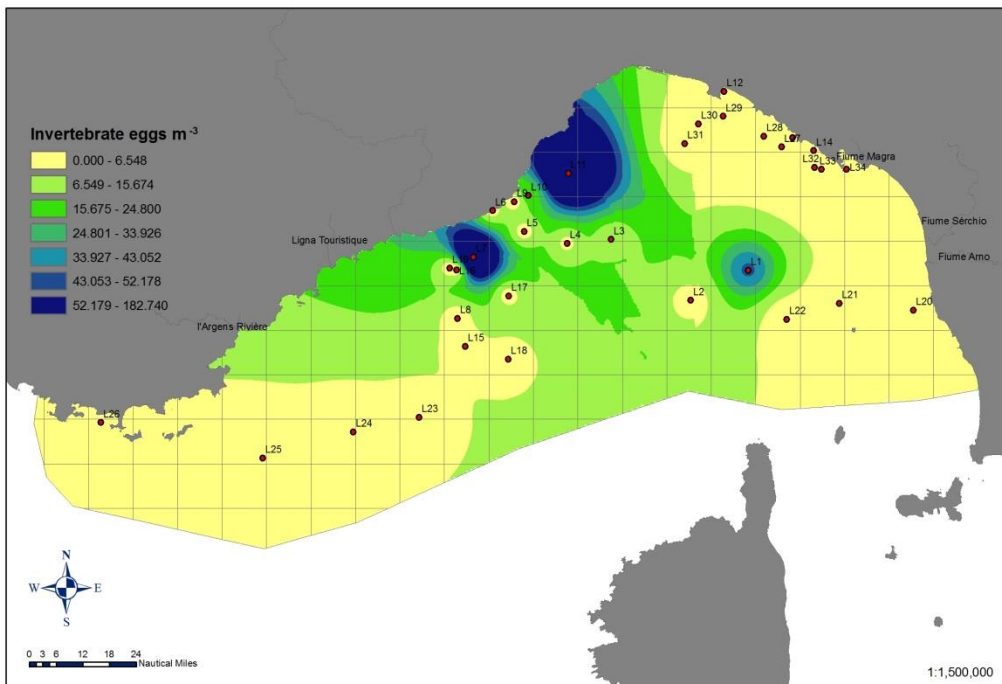
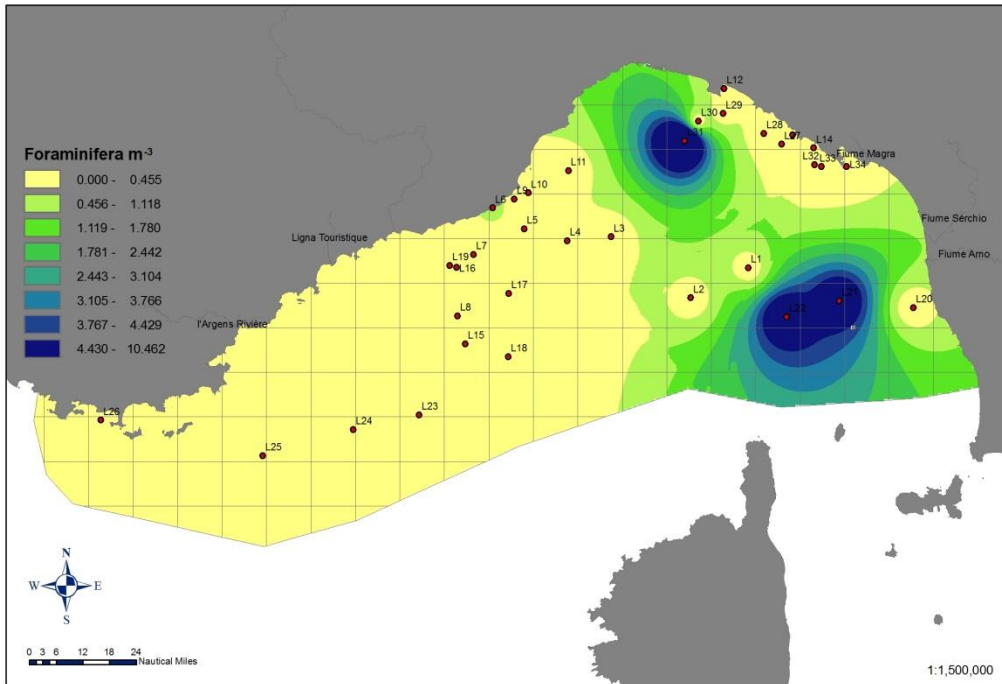
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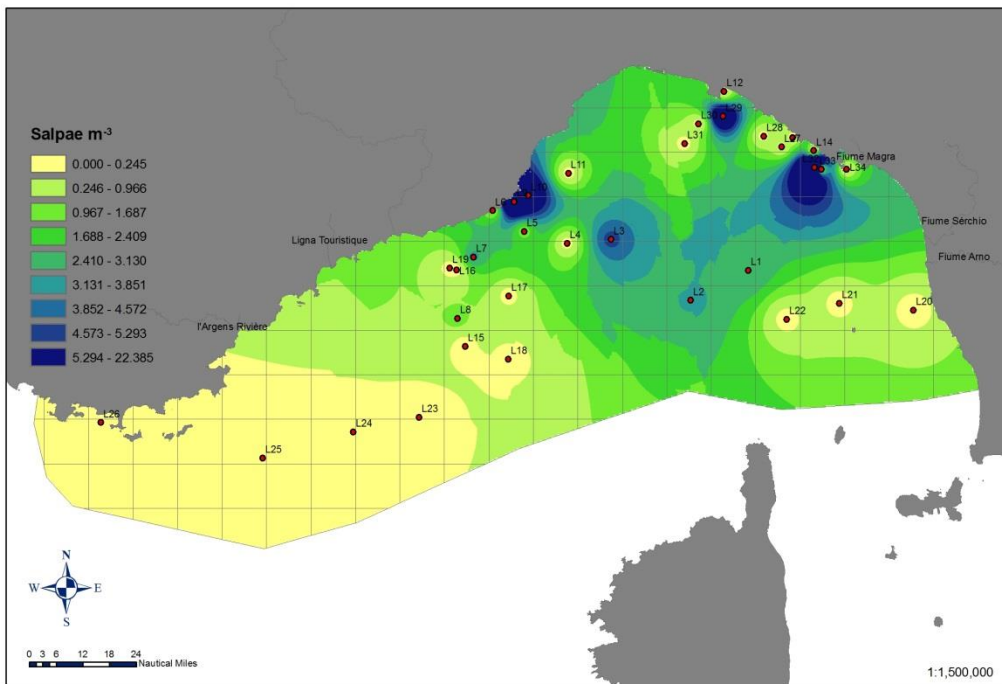
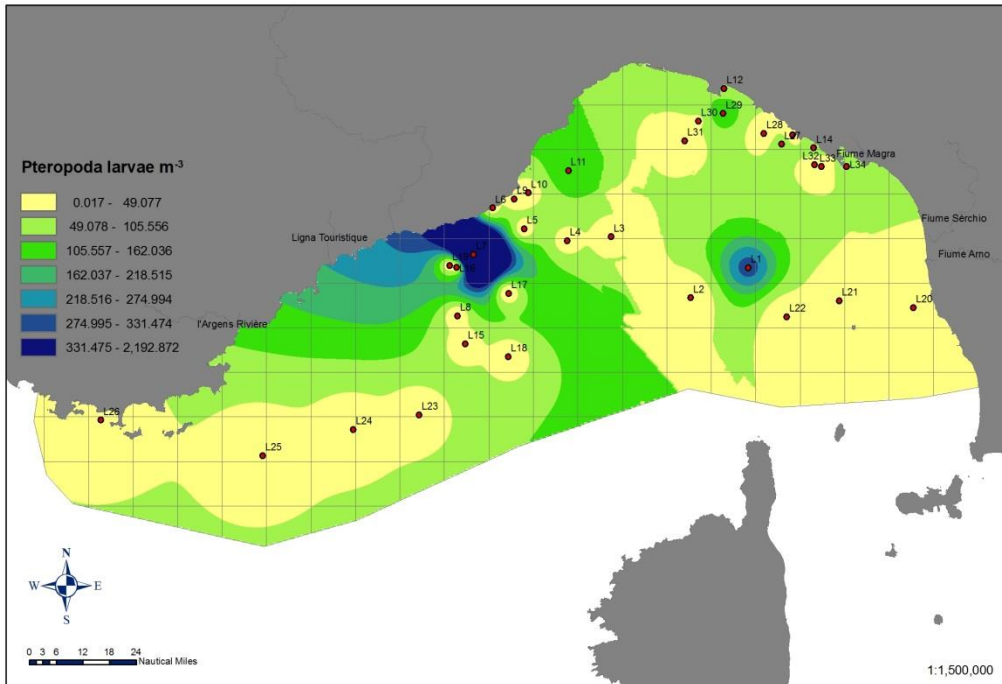
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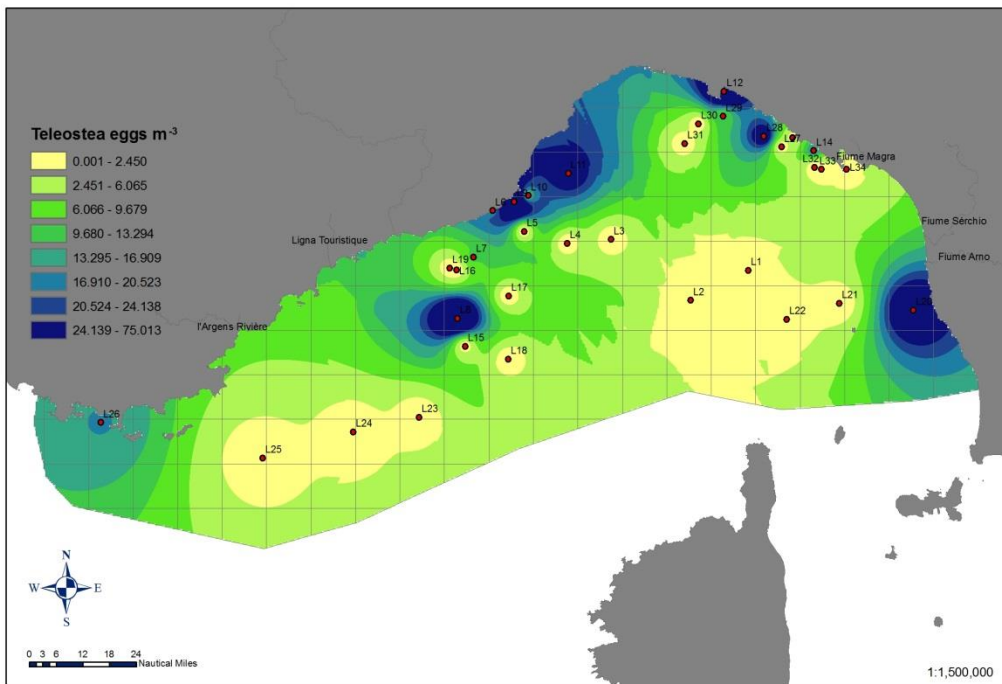
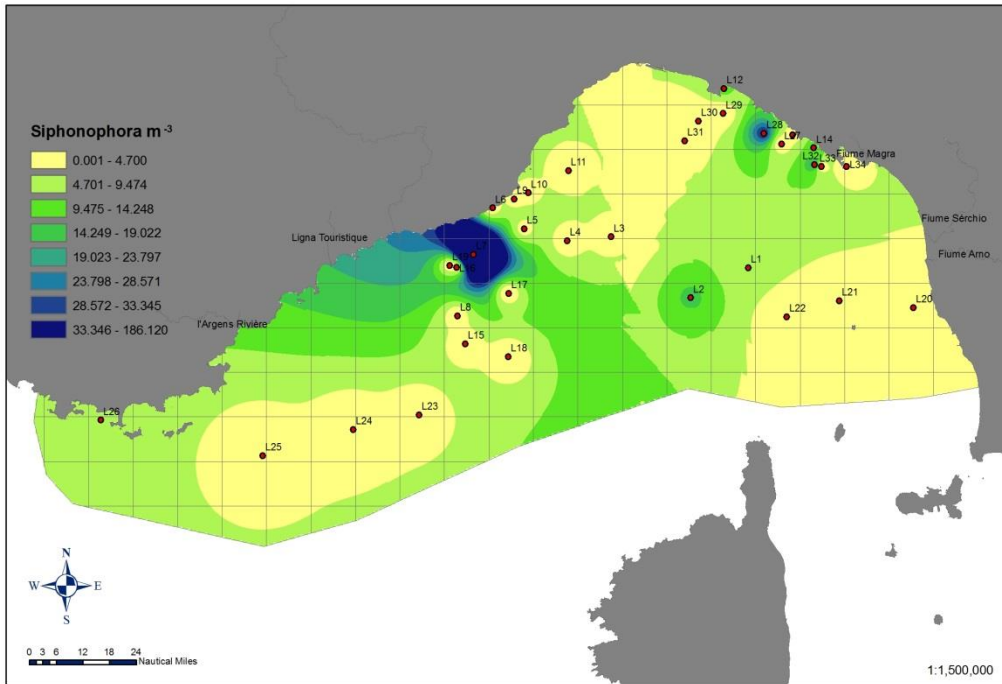












Annesso 2

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Are baleen whales exposed to the threat of microplastics? A case study of the Mediterranean fin whale (*Balaenoptera physalus*)

Maria Cristina Fossi^{a,*}, Cristina Panti^b, Cristiana Guerranti^a, Daniele Coppola^a, Matteo Giannetti^{a,b}, Letizia Marsili^a, Roberta Minutoli^c

^a Department of Environmental Sciences, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy

^b Department of Evolutionary Biology, University of Siena, Via A. Moro 2, 53100 Siena, Italy

^c Department of Animal Biology and Marine Ecology, University of Messina, Viale F. Stagno D'Alcontres, 31, 98166 Messina, Italy

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ABSTRACT

Baleen whales are potentially exposed to micro-litter ingestion as a result of their filter-feeding activity. However, the impacts of microplastics on baleen whales are largely unknown. In this case study of the Mediterranean fin whale (*Balaenoptera physalus*), we explore the toxicological effects of microplastics on mysticetes. The study included the following three steps: (1) the collection/count of microplastics in the Pelagos Sanctuary (Mediterranean Sea), (2) the detection of phthalates in surface neustonic/planktonic samples, and (3) the detection of phthalates in stranded fin whales. A total of 56% of the surface neustonic/planktonic samples contained microplastic particles. The highest abundance of microplastics (9.63 items/m³) was found in the Portofino MPA (Ligurian Sea). High concentrations of phthalates (DEHP and MEHP) were detected in the neustonic/planktonic samples. The concentrations of MEHP found in the blubber of stranded fin whales suggested that phthalates could serve as a tracer of the intake of microplastics. The results of this study represent the first warning of this emerging threat to baleen whales.

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1. Introduction

The emerging issue of microplastics (plastic fragments smaller than 5 mm) in the marine environment has recently received increasing attention (Hidalgo-Ruz et al., 2012). This ubiquitous, persistent form of micro-debris requires centuries to degrade completely. Microplastics are primarily the result of the degradation of plastics released into the environment since the beginning of the plastic age. Micro-debris floating in the Mediterranean Sea has reached maximum levels of 892,000 particles/km². Recently, Collignon et al. (2012) determined neustonic microplastic and zooplankton abundance in the northwestern Mediterranean Sea and showed that the estimated mean abundance of microplastics was of the same order of magnitude as that found for the North Pacific Gyre (0.334 particles/m², Moore et al., 2001), underscoring the high level of this emerging threat in the Mediterranean environment.

Microplastics accumulate at the sea surface, especially within the neustonic habitat (Ryan et al., 2009). This habit harbors a specifically adapted zooplankton fauna. There is increasing concern that a wide range of marine organisms are affected by plastic wastes in the sea. However, the mechanical, physical and toxico-

logical impacts of these wastes are largely unknown. More than 180 species, including planktophagous species, have been shown to absorb plastic debris. Macrodebris ingestion and entanglement are well documented in sea birds, mammals and turtles and more recently in fishes (planktivorous and benthophagous) and invertebrates (Robards et al., 1995; Derraik, 2002; Thompson et al., 2004; Ryan et al., 2009; Boerger et al., 2010; Collignon et al., 2012; Posatto et al., 2011; Dantas et al., 2012; Murray and Cowie, 2011).

No information has previously been reported on the impacts of microplastics on baleen whales, such as fin whales (*Balaenoptera physalus*). The filter-feeding activities of these whales represent a potential source of exposure to micro-litter ingestion. The fin whale, the only resident mysticete in the Mediterranean Sea, forms aggregations during the summer on the feeding grounds of the Pelagos Sanctuary Marine Protected Area (MPA) (Notarbartolo di Sciarra et al., 2003). These whales feed primarily on planktonic euphausiid species. With each mouthful, the whales can trap approximately 70,000 l of water, and their feeding activities include surface feeding. They could therefore face risks caused by the ingestion and degradation of microplastics. Micro-debris can be a significant source of lipophilic chemicals (primarily persistent organic pollutants – POPs) and a source of pollutants such as polyethylene, polypropylene and, particularly, phthalates. These chemical pollutants can potentially affect organisms (Teuten et al., 2007), are potential endocrine disruptors and can affect population viability. With their long lifespan, whales could be chronically

* Corresponding author. Tel.: +39 0577 232913; fax: +39 0577 232930.

E-mail addresses: fossi@unisi.it (M.C. Fossi), panti4@unisi.it (C. Panti), guerrantici@unisi.it (C. Guerranti), coppola32@unisi.it (D. Coppola), giannetti12@unisi.it (M. Giannetti), letizia.marsili@unisi.it (L. Marsili), rminutoli@unime.it (R. Minutoli).

exposed to these persistent contaminants derived from the ingestion and degradation of microplastics.

One toxicological feature of the marine environment that can affect filter-feeding organisms is the influence that microplastics may produce by enhancing the transport and bioavailability of persistent, bioaccumulative and toxic substances. In fact, chemicals for which the logarithm of the octanol/water partitioning coefficient ($K(OW)$) > 5 can potentially be partitioned >1% to polyethylene, a major component of microplastics. Moreover, contaminants such as phthalates and polycyclic aromatic hydrocarbons (PAHs) are among the principal constituents of plastics. The dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid, commonly known as phthalates, are high-production-volume synthetic chemicals; moreover, they are not covalently bound to plastic and migrate from the products to the environment, thus becoming ubiquitous contaminants (Latini et al., 2009). Public and scientific concern about the potential human and wildlife health risks associated with exposure to phthalates has increased in recent years. The primary focus has moved away from the hepatotoxic effects to the endocrine-disrupting potency of these chemicals (Latini, 2005), which have been shown to be reproductive toxicants in animals (Borch et al., 2006). Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment. In both invertebrates and vertebrates, DEHP is rapidly metabolized in the form of its primary metabolite, MEHP (mono-(2-ethylhexyl) phthalate) (Barron et al., 1989), which can be used as a marker of exposure to DEHP.

This case study examines the Mediterranean fin whale, one of the largest filter feeders in the world. This study is the first investigation of the potential impact of microplastics in a baleen whale and suggests the use of phthalates as a tracer of the intake of microplastics through the ingestion of micro-debris and plankton.

2. Methodology

The study included the following three steps: (1) the collection, counting and sorting of microplastics and planktonic organisms in surface neustonic/planktonic and water column samples from the Pelagos Sanctuary MPA (NW Mediterranean Sea); (2) the measurement of phthalate concentrations in surface neustonic/planktonic and water column samples; and (3) the measurement of phthalate concentrations in stranded fin whale specimens collected on the coasts of Italy.

2.1. Step I: collection and sorting of microplastics in surface neustonic/planktonic and water column samples in the Pelagos Sanctuary

Surface neustonic/planktonic and water column samples were collected in the Ligurian Sea and Sardinian Sea (Fig. 1a) in summer 2011 (June–July) during the day with a WP2 standard net (57 cm mouth diameter, 200 μ m mesh size) equipped with a flowmeter for the measurement of the filtered volumes. For each surface sample ($n = 23$; MPM3–MPM26), the net was towed horizontally just below the water surface at a speed of approximately 1 knot for 15 min. For each water column sample (MPP3, MPP10 and MPP22, corresponding to the same geographical coordinates as MPM3, MPM10 and MPM22) (Fig. 1a), the same net was vertically towed from a depth of 50 m to the surface at a speed of 1 m/s. In both cases, the net was washed on board, and each 2-l sample was split into two separate aliquots of 1 l each with a Folsom splitter. One 1-l aliquot was filtered on a 200 μ m mesh sieve and immediately frozen in liquid nitrogen for the subsequent analysis of phthalates. The second aliquot was preserved in 4% formaldehyde-seawater buffered solution for subsequent qualitative analyses. A total of 26 frozen and preserved samples were used for

this study. For the analysis of plankton and plastic particles, the samples were observed under a Leica Wild M10 stereomicroscope. The organisms were counted and taxonomically classified (Table 1, Supplementary data). The plastic particles were counted and measured, and those smaller than 5 mm were classified as microplastics. All the data were normalized to the total volume filtered and expressed as individuals and items/m³. To compare the data with data expressed as items/m² in the literature, the present data can be converted by multiplying the values (items/m³) by 0.5 m, the thickness of the water stratum sampled with the WP2 net as described above.

2.2. Step II: detection of phthalates in surface neustonic/planktonic and water column samples

DEHP and MEHP were analyzed in the surface neustonic/planktonic and water column samples (0.5–0.7 g) from the two sampling sub-areas (Ligurian Sea and Sardinian Sea) following a method described by Takatori et al. (2004), with a few modifications described in Guerranti et al. (2012). Each sample was thawed and weighed, and acetone was added. The sample obtained in this way was sonicated. The organic part, containing DEHP and MEHP, was separated from the remaining water, and the supernatant was isolated. The supernatant phase was then recovered and combined with that resulting from the first extraction and was then evaporated in a centrifugal evaporator. The extract was then resuspended with 0.5 ml of acetonitrile and passed through a nylon filter with pores of 2 μ m. Subsequently, the sample was placed in an autosampler vial and injected into an LC-ESI-MS system. The instrumental analysis was performed with a Finnigan LTQ Thermo LC/MSn 110 with an ESI interface. A total of 5 μ l of the extracted sample was injected via the autosampler into the HPLC system. A reverse-phase HPLC column (Wakosil 3C18, 2.0 \times 100 mm, 3 μ m; Wako Pure Chemical Industries Ltd.) was used. The mobile phases consisted of 100% acetonitrile (A) and 0.05% aqueous acetic acid (B). Elution was performed using an isocratic mode (A/B: 15/85, v/v) at 0.25 ml/min. ESI-MS was operated in the negative or positive ion mode depending on the analytes (MEHP was detected in the negative mode, whereas DEHP was detected in the positive mode). The heated capillary and voltage were maintained at 500 °C and ± 4.0 kV, respectively. The ions used for identification were (parent ion/daughter ion) 277/134 and 391/149 for MEHP and DEHP, respectively. For the quantitative analysis, a four-point calibration curve prepared by the progressive dilution of a solution of the two analytes of interest was used. Blanks were analyzed with each set of five samples as a check for possible laboratory contamination and interference. The data quality assurance and quality control protocols also included matrix spikes and continuing calibration verification. The limits of detection (LODs) and limits of quantification (LOQs) for the compounds analyzed were the values of the compound in the +3 SD and +10 SD blanks, respectively. The LOD and LOQ were 1 and 2 ng/g, respectively, for MEHP and 5 and 10 ng/g, respectively, for DEHP.

The levels of analytes below the limits of detection (<LOD) were specified as values equal to the value of the LOD. If the analyte was present at levels between the LOD and the LOQ, the LOQ value was used. The values are expressed as fresh weight (f.w.).

2.3. Step III: measurement of phthalate concentrations in stranded fin whale specimens collected along the coasts of Italy

Blubber samples were collected close to the dorsal fin in five stranded fin whales (sub-adults and adults) during the period July 2007–June 2011 at five different sites on the Italian coast. The samples were stored at -20 °C prior to analysis. The details of the location and gender of the stranded whales are shown in Fig. 1b. DEHP

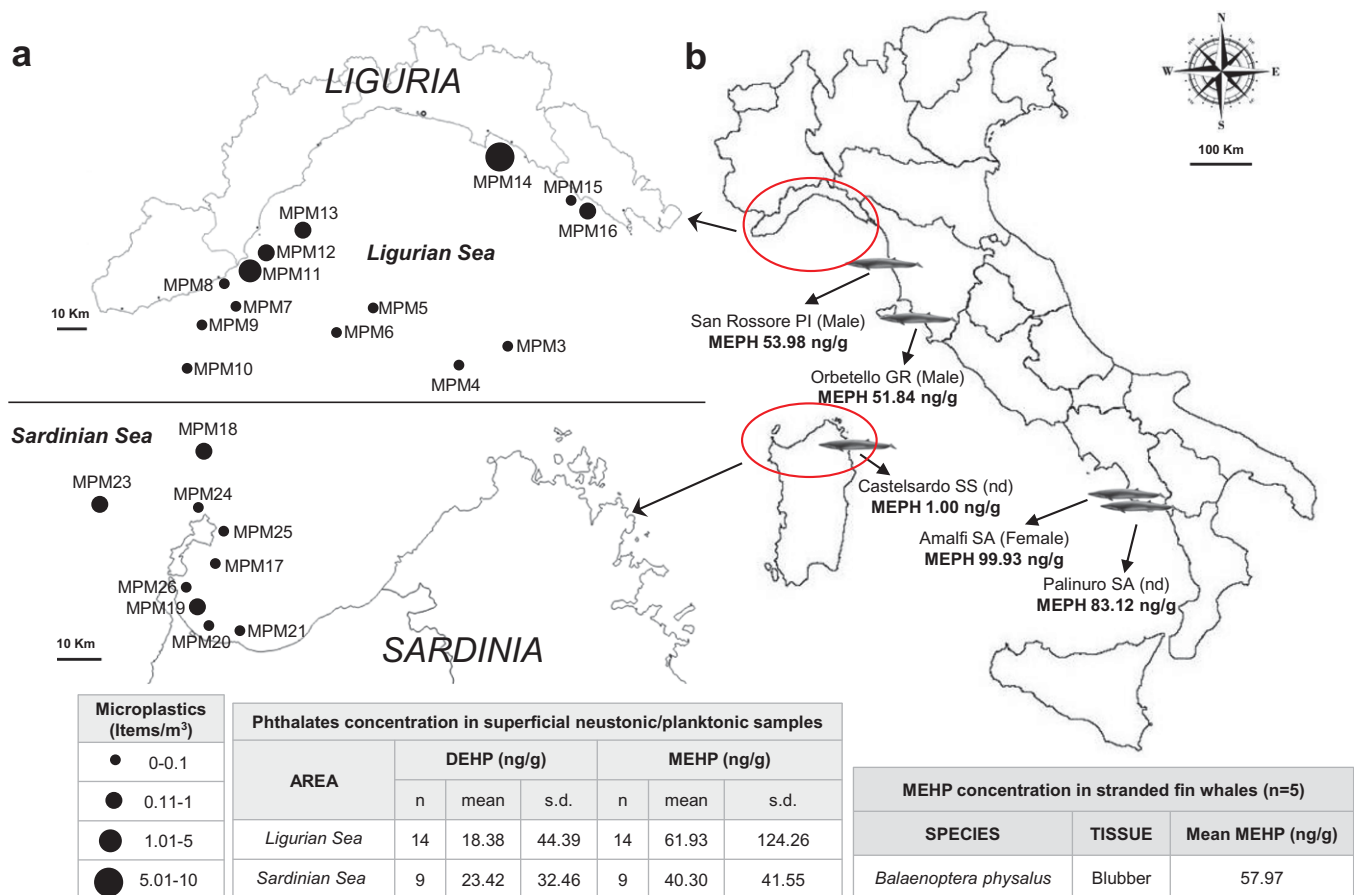


Fig. 1. (a) Microplastic particles in superficial neustonic/planktonic samples (items/m³) collected in the Pelagos Sanctuary (Ligurian Sea and Sardinian Sea) and mean DEHP and MEHP concentrations (ng/g). Geographical coordinates of sampling sites are reported in Table 2 of Supplementary data. (b) DEHP concentrations (ng/g) in blubber samples of five stranded fin whales collected along the Italian coasts during the period July 2007–June 2011 in five different locations.

Table 1
Microplastic particles in superficial neustonic/planktonic samples (items/m³) collected in the Pelagos Sanctuary, zooplankton abundance (ind/m³), DEHP and MEHP concentrations (ng/g f.w.), mean values \pm S.D. (see Fig. 1 for sampling sites).

| Sample | Items/m ³ | Zooplankton abundance (ind/m ³) | DEHP (ng/g) | MEHP (ng/g) |
|----------------------|-----------------------------------|---|-------------------------------------|--------------------------------------|
| <i>Ligurian Sea</i> | | | | |
| MPM3 | 0.00 | 403.96 | 5.00 | 1.00 |
| MPM4 | 0.10 | 167.78 | 5.00 | 55.20 |
| MPM5 | 0.10 | 23.45 | 10.00 | 1.00 |
| MPM6 | 0.00 | 43.67 | 172.41 | 3.12 |
| MPM7 | 0.00 | 36.77 | 5.00 | 5.75 |
| MPM8 | 0.05 | 204.71 | 5.00 | 454.07 |
| MPM9 | 0.00 | 4275.51 | 5.00 | 1.00 |
| MPM10 | 0.00 | 193.15 | 5.00 | 2.00 |
| MPM11 | 1.35 | 377.49 | 5.00 | 37.64 |
| MPM12 | 0.50 | 496.35 | 5.00 | 4.87 |
| MPM13 | 0.33 | 6147.00 | 10.00 | 1.00 |
| MPM14 | 9.67 | 4253.33 | 10.00 | 188.94 |
| MPM15 | 0.04 | 179.51 | 10.00 | 25.68 |
| MPM16 | 0.95 | 4645.71 | 5.00 | 85.78 |
| Mean | 0.94 \pm 2.55 | 1532.03 | 18.38 \pm 44.39 | 61.93 \pm 124.26 |
| <i>Sardinian Sea</i> | | | | |
| MPM17 | 0.00 | 82.74 | 76.02 | 19.83 |
| MPM18 | 0.83 | 27.07 | 10.00 | 1.00 |
| MPM19 | 0.11 | 744.54 | 10.00 | 11.30 |
| MPM20 | 0.00 | 668.66 | 5.00 | 107.11 |
| MPM21 | 0.03 | 90.19 | 10.00 | 35.56 |
| MPM23 | 0.24 | 102.73 | 5.00 | 1.00 |
| MPM24 | 0.00 | 523.27 | 84.81 | 109.93 |
| MPM25 | 0.00 | 15000.00 | 5.00 | 30.64 |
| MPM26 | 0.00 | 3919.72 | 5.00 | 46.34 |
| Mean | 0.13 \pm 0.27 | 2350.99 | 23.42 \pm 32.46 | 40.30 \pm 41.55 |
| Total Mean | 0.62 \pm 2.00 | 1852.49 | 20.36 \pm 39.42 | 53.47 \pm 99.34 |

and MEHP were extracted from blubber (1 g), and phthalate concentrations were measured with the method described above.

3. Results

Of the 23 surface neustonic/planktonic samples, 13 contained plastic particles (Table 1, Fig. 1a). The highest microplastic abundance (9.67 items/m³, equivalent to 4.83 items/m²) was found in a sample collected near the Portofino MPA (Ligurian Sea). Large amounts of plastic particles were detected in the surface neustonic/planktonic samples collected in the Pelagos Sanctuary areas investigated (mean value 0.62 items/m³). The amounts of plastic particles were approximately seven times higher in the samples from the Ligurian Sea (mean value 0.94 items/m³) than in the samples from the Sardinian Sea (mean value 0.13 items/m³) (Table 1). Plastic particles were not found in the three water column samples (Table 2). The planktonic species were taxonomically determined, and the results are shown in Table 1 of Supplementary data.

High concentrations of the phthalates MEHP and DEHP were detected for the first time in the surface neustonic/planktonic samples collected in the Pelagos Sanctuary areas. The values of MEHP were approximately 1.5 times higher in the samples from the Ligurian Sea than in the samples from the Sardinian Sea. Lower concentrations of MEHP were detected in the 3 water column samples than in the surface samples (Table 2).

The presence of harmful chemicals in Mediterranean fin whales, associated with the potential intake of plastic derivatives by water filtering and plankton ingestion, was demonstrated for the first time by the results of this study, which documented the presence of relevant concentrations of MEHP in the blubber of four out of five stranded fin whales (Fig. 1b). MEHP is a marker for exposure to DEHP, whereas DEHP was never detected in the samples. It is not surprising that DEHP was not detected in these samples, as it is well known that the DEHP is rapidly metabolized to MEHP, its primary metabolite (Latini et al., 2004). The preliminary data obtained by the current study suggest that phthalates can serve as a tracer of the intake of microplastics by fin whales resulting from the ingestion of micro-litter and plankton.

4. Discussion

The present study, following the recent publication by Collignon et al. (2012), provides an initial insight into microplastic pollution in the Mediterranean Sea by reporting the concentrations and spatial distribution of microplastics in the area of Pelagos Sanctuary. We emphasize that the mean abundance of microplastics estimated in this study is of the same order of magnitude as that found for the North Pacific Gyre (Collignon et al., 2012), suggesting the high level of this emerging threat in the only pelagic MPA of the Mediterranean Sea.

The Pelagos Sanctuary for Mediterranean Marine Mammals is a marine protected area of approximately 90,000 km² in the north-western Mediterranean Sea. A remarkable cetacean fauna consisting of 8 species, including the baleen whale *B. physalus*, coexists in the Sanctuary with very high levels of human pressure. Plastic

from coastal tourism, recreational and commercial fishing, marine vessels and marine industries can directly enter the marine environment and pose a risk to biota both as macroplastics and, following long-term degradation, as microplastics. Within the Pelagos Sanctuary, the Portofino MPA showed the highest values of microplastic items/m³. This area was also confirmed as a “hot spot” for microplastics by Collignon et al. (2012). These results serve to focus particular attention on the conservation status of an area with a high level of exploitation by tourists and on the balance between conservation measures and management.

Previously, very few studies have addressed the impact of microplastics on filter-feeding organisms or other planktivorous animals. No previous studies have assessed the potential impact of microplastics on large filter-feeding organisms, such as baleen whales.

At the lowest level of the food web, the great abundance of microplastics in the photic zone could both interfere with and be a severe threat to plankton viability. Microplastic debris has been found in the gastrointestinal tracts of several planktivorous fishes (*Myctophidae*, *Stomiidae* and *Scorpaenidae*) in the North Pacific Gyre (Boerger et al., 2010). In the Mediterranean Sea, during the survey recently carried out by Collignon et al. (2012), plastic micro-debris was found in the stomachs of myctophids (*Myctophum punctatum*). Moreover, several studies report the ingestion of plastic debris of different sizes, colors and shapes by both epibenthic and hyperbenthic fish species (*Ariidae*, *Scianidae*) inhabiting a demersal estuarine environment in the tropical Western South Atlantic (Costa et al., 2011; Possatto et al., 2011; Dantas et al., 2012). The occurrence of interactions between several species of marine mammals and marine debris (Williams et al., 2011) and of plastic ingestion in Franciscana dolphins were also recently reported (Denuncio et al., 2011). However, the physiological and toxicological effects of plastic ingestion by filter-feeding organisms are poorly investigated and understood, as are the implications of plastic ingestion occurring through the food chain.

Marine plastics have been found to adsorb and transport chemicals, including high concentrations of organochlorines such as polychlorinated biphenyls (PCBs), dichlorodiphenyl trichloroethane (DDT) and PAHs (Teuten et al., 2007). After the ingestion of plastics by an organism, the presence of digestive surfactants is known to increase the bioavailability of these compounds sorbed to plastics (Voparil and Mayer, 2000) by markedly increasing the desorption rate of plastics compared with that found in sea water (Teuten et al., 2007). Due to the large surface-area-to-volume ratio of microplastics, marine organisms may be particularly at risk of exposure to leached additives after microplastics are ingested. Such additives may interfere with biologically important processes, potentially resulting in endocrine disruption (Barnes et al., 2009; Lithner et al., 2009, 2011). In this context, it is known that commonly used additives, such as brominated flame retardants, phthalates and the constituent monomer bisphenol A, can act as endocrine-disrupting chemicals because they can mimic, compete with or disrupt the synthesis of endogenous hormones (Talsness et al., 2009). In particular, phthalates have been associated with a range of molecular, cellular and organ effects in aquatic invertebrates and fish (Oehlmann et al., 2009). Bisphenol A is both

Table 2

Microplastic particles in water column samples (items/m³) collected in the Pelagos Sanctuary, zooplankton abundance (ind/m³), DEHP and MEHP concentrations (ng/g f.w.), mean values ± S.D (see Fig. 1 for sampling sites).

| Sample | Items/m ³ | Zooplankton abundance (ind/m ³) | DEHP (ng/g) | MEHP (ng/g) |
|-------------|----------------------|---|--------------------|--------------------|
| MPP3 | 0.00 | 49.71 | 5.00 | 1.00 |
| MPP10 | 0.00 | 1266.05 | 5.00 | 4.32 |
| MPP22 | 0.00 | 864.88 | 5.00 | 1.00 |
| Mean | 0.00 | 726.88 | 5.00 ± 0.00 | 2.11 ± 1.92 |

an estrogen agonist and an androgen antagonist, and it can differentially affect reproduction and development, depending on its concentration and the species affected. Nevertheless, Oehlmann et al. (2009) note that there has been relatively little research into the chronic effects of long-term exposure to these additives in aquatic organisms.

The present data represent the first evidence of the potential impact of the most abundant plastic derivatives (phthalates) in a baleen whale, the second-largest filter feeder in the world: the Mediterranean fin whale. The fin whale is a cosmopolitan cetacean. It is found in the largest water masses of the world, from the equator to the polar regions. Despite its cosmopolitan distribution, it is classified as Endangered on the IUCN Red List. In general, rorqual feeding has been described as the largest biomechanical event that has ever existed on Earth (Croll and Tershy, 2002). Fin whales capture food by initially swimming rapidly toward a school of prey and then decelerating while opening the mouth to gulp vast quantities of water and schooling prey. Fin and blue whales foraging on krill off the coast concentrate their foraging effort on dense aggregations of krill (150–300 m) in the water column during the day and feed near the surface at night (Croll et al., 2005).

It is well known that the fin whale in the Mediterranean Sea feeds preferentially on the planktonic euphausiid *Meganyctiphanes norvegica*. Nevertheless, depending on the area and the season, the whale feeds on a wide spectrum of marine organisms, including copepods, other euphausiid species (e.g., *Thysanoessa inermis*, *Calanus finmarchicus*, *Euphausia krohni*) and small schooling fish (Orsi Relini and Giordano, 1992; Relini et al., 1992; Notarbartolo di Sciarra et al., 2003). With each mouthful, a fin whale can trap approximately 70,000 l of water. For this reason, a whale could risk ingesting a great amount of microplastic debris, both directly from the water and indirectly from the plankton (during both surface feeding and deeper feeding activity). After microplastics are ingested, a fin whale may be exposed directly to leached additives, such as polybrominated diphenyl ethers, phthalates and bisphenol A and their potential toxicological effects.

Preliminary data on MEHP in 5 samples of *Euphausia krohni* collected in the Sicilian Channel reported high concentrations of this contaminant ranging from 8.35 to 51.14 ng/g. These results suggested that plastic derivatives also occur in planktonic species inhabiting the water column (unpublished data, Guerranti personal communication).

In view of the presence of microplastics in the Mediterranean environment, the detection of plastic additives in the blubber of fin whales and the long lifespan of the species, fin whales appear to be chronically exposed to persistent and emerging contaminants as a result of microplastic ingestion. In this context, the preliminary observations presented in this paper suggest that phthalates can serve as a tracer for the intake of microplastics in micro-litter and in plankton by fin whales. These observations represent a warning that the endangered Mediterranean population of this baleen whale is exposed to endocrine disruptors such as MEHP. The results of this study are consistent with the evidence previously reported by Fossi et al. (2010) of an early warning signal of endocrine interference furnished by the up-regulation of the estrogen receptor alpha gene detected in skin biopsies of male Mediterranean fin whales compared with fin whales from the Sea of Cortez (Mexico). This “undesirable biological effect” (in agreement with the description of the concept of biomarkers in Descriptor 8 of the Marine Strategy Framework Directive) can suggest that the Mediterranean population is exposed to a mixture of persistent and emerging contaminants, such as endocrine disruptors, that may impair the population viability of this already endangered species.

In this context, surveys covering much of the western Mediterranean basin have estimated the fin whale population to be 3,583 individuals (Forcada et al., 1996), 901 of which inhabit the

Corsican-Ligurian-Provencal basin (Forcada et al., 1995). However, according to more recent data on the Pelagos Sanctuary, the estimated population has decreased markedly (approximately by a factor of six) in the past 20 years (Panigada et al., 2011) raising particular concerns about the status of this species.

In conclusion, the present data represent the first evidence of the potential impact of plastic additives (phthalates) in baleen whales. These results underscore the importance of future research on the detection of the toxicological impact of micro-plastics in filter-feeding species such as mysticete cetaceans, the basking shark and the devil ray. The results also underscore the potential use of these species in the implementation of Descriptor 10 (marine litter) in the EU Marine Strategy Framework Directive as indicators of the presence and impact of micro-litter in the pelagic environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.marpolbul.2012.08.013>.

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Large filter feeding marine organisms as indicators of microplastic in the pelagic environment: The case studies of the Mediterranean basking shark (*Cetorhinus maximus*) and fin whale (*Balaenoptera physalus*)

Maria Cristina Fossi^{a,*}, Daniele Coppola^a, Matteo Baini^a, Matteo Giannetti^{a,b},
Cristiana Guerranti^a, Letizia Marsili^a, Cristina Panti^a, Eleonora de Sabata^c, Simona Clò^{c,d}

^a Department of Physical, Earth and Environmental Sciences, University of Siena, Via P.A. Mattioli 4, 53100 Siena, Italy

^b Department of Life Sciences, University of Siena, Via A. Moro 2, 53100 Siena, Italy

^c MedSharks, Via Ruggero Fauro 82, 00197 Rome, Italy

^d CTS, via Albalonga 3, 00183 Roma, Italy

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ABSTRACT

The impact of microplastics (plastic fragments smaller than 5 mm) on large filter feeding marine organisms such as baleen whales and sharks are largely unknown. These species potentially are ingesting micro-litter by filter feeding activity. Here we present the case studies of the Mediterranean fin whale (*Balaenoptera physalus*) and basking shark (*Cetorhinus maximus*) exploring the toxicological effects of microplastics in these species measuring the levels of phthalates in both species. The results show higher concentration of MEHP in the muscle of basking shark in comparison to fin whale blubber. These species can be proposed as indicators of microplastics in the pelagic environment in the implementation of Descriptor 8 and 10 of the EU Marine Strategy Framework Directive (MSFD).

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1. Introduction

Are the largest filter feeder marine organisms affected by any of the smallest human debris? How can 5 mm plastic debris affect 24 m long marine mammals and 7 m long sharks? In this paper we investigate the invisible war between the Mediterranean fin whale (*Balaenoptera physalus*) and basking shark (*Cetorhinus maximus*) against the smallest marine debris and their potential toxicological effects. Why microplastics may pose a threat to these species?

In 2009, 230 million tons of plastics were produced globally, Europe is the second larger producer of plastic (PlasticsEurope, 2012). According to sea-based sources such as shipping, fishing and transport activities (Derraik, 2002) and land-based sources such as tourism, adjacent industries or river inputs (Browne et al., 2010), plastics are entering our seas and oceans, “posing a

complex and multi-dimensional challenge with significant implications for the marine and coastal environment and human activities all over the world” (UNEP, 2009).

For the Mediterranean environment marine litter (including plastic) represents a serious concern (UNEP, 2009; UNEP/MAP, 2011; MSFD, 2011). Three billions of litter items float or cover the sea bottom in the Mediterranean Sea, which 70–80% is plastic waste. The increasing of marine litter is mainly related to waste production in-land with an average amount of municipal solid waste in the EU of 520 kg per person/year and a projected increase to 680 kg per person/year by 2020.

The incidence of debris in the marine environment is cause for concern. It is known to be harmful to marine organisms and to human health (Derraik, 2002; Gregory, 2009; Wright et al., 2013), it represents a hazard to maritime transport, it is aesthetically detrimental, and may also have the potential to transport contaminants (Mato et al., 2001; Teuten et al., 2009). Marine debris, and in particular the accumulation of plastic debris, has been identified as a global problem alongside other key issues such as

* Corresponding author. Tel.: +39 0557 232883; fax: +39 0557 232930.
E-mail address: fossi@unisi.it (M.C. Fossi).

climate change, ocean acidification and loss of biodiversity. Impacts vary depending on the type and size of debris and the organisms affected.

The occurrence of microplastics (MPs – generally defined as fragments less than 5 mm in dimension – NOAA) in the ocean is an emerging world-wide concern. Due to high sorption capacity of plastics for hydrophobic organic chemicals, the adherent chemicals can be transported by MPs traveling long distances (Lee et al., 2013). MPs can serve as carrier of persistent organic pollutants (POPs) in marine ecosystems (Rochman et al., 2013; Koelmans et al., 2013). Small plastic particles in the environment are of particular concern as a wide range of organisms, from plankton to larger vertebrates such as turtles or whales, may ingest them (Wright et al., 2013). In particular, while evidence of macro and microplastic negative effects on marine organisms is growing, little scientific investigation has focused on the problem in the Mediterranean. More information is required about plastic and microplastic inputs, spatial and temporal distributions, including transport dynamics, interactions with biota and potential accumulation areas.

Microplastics found in the marine environment are likely to be derived either directly or through the fragmentation of larger items. MPs can be subdivided by usage and origin as: i) Primary, pellets used in the plastics industry, and in certain applications such as abrasives; ii) Secondary, fragments resulting from the degradation and breakdown of larger items.

Microplastics floating over water are transported by ocean currents and are found in regions where water circulation is relatively stationary or on sea shores (Hidalgo-Ruz et al., 2012). A number of heavily produced low density plastics (e.g. polypropylene, polyethylene, and polystyrene) have been identified as the main components of MPs, and these have various shapes and sizes, ranging from a few micrometers to a few millimeters (Hidalgo-Ruz et al., 2012; Martins and Sobral, 2011).

Microplastics are accumulating at the sea surface, especially within the neustonic habitat (Ryan et al., 2009) that included a specifically adapted zooplankton fauna. Basking shark and particularly fin whale, being characterized by a long life span, could be chronically exposed to these persistent contaminants both leaching from microplastic ingestion and degradation and through the food chain.

Recent studies have identified potential effects of plastic particles mainly in invertebrates and fish, including: I) transport of persistent, bioaccumulating and toxic (PBT) substances from plastics; II) leaching of additives such as phthalates from the plastics; III) physical harm (Wright et al., 2013).

However, there is still little monitoring data on the occurrence of microplastics in large marine vertebrates. Until the paper of Fossi et al. (2012), no data were reported on the impacts of microplastics on large filter feeding marine organisms such as baleen whales or sharks. These species potentially undergo to the ingestion of micro-litter by filtering feeding activity.

In this paper we focus on the case study of the two large Mediterranean filter feeders, the fin whale and basking shark.

The basking shark (*C. maximus* Gunnerus, 1765) is a very large, filter-feeding cold-water and migratory pelagic species. It is widely distributed throughout temperate waters but only regularly seen in few favored coastal locations. It may be considered frequently present in the Mediterranean, especially in the North-Western part, mainly in spring (Mancusi et al., 2005). Basking sharks are regular seasonal visitors in coastal waters of Sardinia, where between 2005 and 2012 a total of 111 individuals (including 14 captures) were recorded within “Operazione Squalo Elefante”, the first dedicated basking shark research project in the Mediterranean basin (de Sabata and Clò, 2010). The basking shark is one of only three

shark species that filter seawater for planktonic prey. It captures zooplankton by forward swimming with an open mouth, so that water passively flows across the gill-raker apparatus. The rates of gastro-intestinal evacuation in basking sharks are unknown; however, filtration rates have been estimated using measurements of swimming speed and mouth gape area. Seawater filtration rate for a 7 m basking shark (mouth gape area ca. 0.4 m²) swimming at a speed of 0.85 m s⁻¹ was calculated to be 881 m³ h⁻¹; if it fed constantly in food patches, a so 5–7 m long basking shark might consume 30.7 kg of zooplankton in a day (Sims, 2008). During this massive filtering activities the basking shark could undergo to the ingestion and degradation of microplastics.

Due to its slow growth rate, lengthy maturation time, long gestation period, probably low fecundity, probable small size of existing populations – some severely depleted by targeted fisheries – the basking shark is classified by the IUCN Red List of Threatened Species as “Endangered” in the North-East Atlantic Ocean and “Vulnerable” in the Mediterranean Sea (Fowler, 2009; Cavanagh and Gibson, 2007). It is listed in all four major International conventions (Bern, CMS, CITES, Barcelona). Every year basking sharks are accidentally caught in small-scale fisheries throughout the Mediterranean region.

The fin whale (*B. physalus*, Linnaeus 1758), one of the largest filter feeders in the world, feeds primarily on planktonic euphausiid species. This baleen whale, the only resident mysticete in the Mediterranean Sea, forms aggregations during the summer on the feeding grounds of the Pelagos Sanctuary Marine Protected Area (MPA). The fin whale is a wide ranging cetacean. It is found in largest water masses of the world, from the Equator to the polar regions, but, in spite of its cosmopolitan distribution, it is classified as “Endangered” by the IUCN Red List of Threatened Species. Fin whale feeding, in general, has been described as the largest biomechanical event that has ever existed on earth (Croll and Tershy, 2002). Fin whales capture food by initially swimming rapidly at a prey school and then decelerating while opening the mouth to gulp vast quantities of water and schooling prey. Fin and blue whales foraging on krill off the coast, concentrate their foraging effort on dense aggregations of krill (150–300 m) in the water column during the day, and near the surface at night (Croll et al., 2005).

With each mouthful, the fin whales can trap approximately 70,000 l of water. Since their feeding activities include surface feeding and, they undergo to the risk of the ingestion of MPs occurring in the sea surface and consequent degradation once ingested by the organism. Seawater filtration daily is 5893 m³ rate with 913 kg of plankton consumed daily.

One major toxicological aspect of MPs in the marine environment and, consequentially, on filter-feeding organisms, is the influence that microplastics may have on enhancing the transport and bioavailability of PBT persistent, bioaccumulative, and toxic substances. These two large filter feeders species (fin whale and basking shark) could therefore face risks caused by the ingestion and degradation of microplastics.

PBT compounds, such as dichlorodiphenyltrichloroethane (DDT) or polychlorinated biphenyls (PCBs), are of particular concern for human health and the environment. Plastic debris can be a source of PBT chemicals. Some plastic debris can release toxic chemicals that have been added to enhance the performance of the plastic (such as phthalates, nonylphenol, bisphenol A, brominated flame retardants). Plastic debris may also be a sink for toxic chemicals: toxic chemicals from the environment can sorb to the debris and to be released once inside the organism (Engler, 2012; Lithner et al., 2011). Since PBT chemicals, generally, have low solubility in marine water they tend to migrate into water microlayers where they tend to migrate to microdebris or in the sediments also

biomagnifying the concentration and effect in organisms that can ingest MP particles. Furthermore, plastic debris sorbs PCBs and DDE about one hundred times more than naturally suspended organic matter. PCBs and DDE sorb to debris with a partition coefficient, K_d , of approximately 100,000–1,000,000 over seawater. Similarly, phenanthrene, a PAH, partitions to plastic debris 380–13,000-fold over seawater (Engler, 2012).

Most of the chemicals absorbed or added to plastic that can potentially affect organisms (Teuten et al., 2007) have endocrine disruptors potency and affect population viability.

Phthalates are a class of chemicals commonly used to make rigid plastics softer to enhance the use of some plastic polymers. Phthalates generally do not persist in the environment, but may leach from plastic debris on a fairly steady basis. The dialkyl- or alkyl/aryl esters of 1,2-benzenedicarboxylic acid, commonly known as phthalates, are high-production volume synthetic chemicals. They are not covalently bound to plastic so can migrate from the products to the environment, thus becoming ubiquitous contaminants (Latini et al., 2004, 2009). Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment; DEHP, in organisms, both invertebrates and vertebrates, is rapidly metabolized in its primary metabolite, MEHP (mono-(2-ethylhexyl) phthalate) (Barron et al., 1989), that can be used as marker of exposure to DEHP.

Concerning the problem of marine litter in EU waters, the amount of marine litter in the Mediterranean environment and the effects on sentinel organisms need to be reduced to achieve the GES (Good Environmental Status) as planned by the European Marine Strategy Framework Directive (MSFD) by 2020. As an amendment to the MSFD the “composition of micro-particles (in particular microplastics) has to be characterized in marine litter in the marine and coastal environment” (MSFD GES Technical Subgroup on Marine Litter, 2011). Currently, there is a severe gap in establishing the presence and effects of MPs on Mediterranean marine,

which must be done with sentinel species to determine effects and implement future mitigation actions.

Here we present the case studies of the two Mediterranean larger filter feeders, the fin whale (*B. physalus*) and basking shark (*C. maximus*), exploring the toxicological effects of MPs in these species and suggesting the possible implication as indicators of MPs in the pelagic environment in the implementation of the European MSFD. We also suggest the possible implication of considering these species as indicators of MPs in the pelagic environment in the implementation of the European MSFD. We also suggest the use of phthalates and organochlorines in plankton, shark and whale, as a tracer of microplastics assumption by ingestion in these species.

2. Material and methods

This work is implemented through three main steps: 1) detection of phthalates in *Euphausia krohnii*; 2) detection of phthalates and organochlorine compounds (OCs) in accidentally caught basking shark in Italian waters; 3) detection of phthalate and OCs content in stranded fin whale specimens collected on the Italian coasts. Details on gender, size, date and location of the stranded animals are reported in Fig. 2.

2.1. Specimens and sampling sites

Ten pools of 30–40 specimens of the Euphausiidae *E. krohnii* were sampled during two expeditions in collaboration with National Council of Research (CNR) with the Oceanographic ship “Urania” in the Channel of Sicily, South Mediterranean Sea.

Muscle samples were collected from accidentally caught specimens of basking shark in Italian waters within “Operazione Squalo Elefante”, during the period 2007–2013: four in the Pelagos Sanctuary (3 in Sardinia, 1 in the Ligurian Sea) and one off the southern border (Latium), one in Mola di Bari (Puglia) (Fig. 1). Blubber and

| Sample ID | Area | Date | Length (m) | Sex |
|------------------------------|-------------------------|------|------------|-----|
| <i>Balaenoptera physalus</i> | | | | |
| BP 1 | Palinuro (Campania) | 2007 | 13.00 | F |
| BP 2 | Amalfi (Campania) | 2007 | 13.50 | F |
| BP 3 | Giannella (Tuscany) | 2008 | 10.40 | M |
| BP 4 | S. Rossore (Tuscany) | 2011 | 16.70 | M |
| BP 5 | Castelsardo (Sardinia) | 2011 | 8.00 | - |
| <i>Cetorhinus maximus</i> | | | | |
| CM 1 | Capo Figari (Sardinia) | 2006 | 4.50 | F |
| CM 2 | Anzio (Latium) | 2007 | 3.50 | M |
| CM 3 | Cala Gonone (Sardinia) | 2010 | 8.00 | F |
| CM 4 | Ospedaletti (Liguria) | 2010 | 3.90 | M |
| CM 5 | Porto Torres (Sardinia) | 2006 | - | - |
| CM 6 | Mola di Bari (Puglia) | 2013 | 7.00 | M |

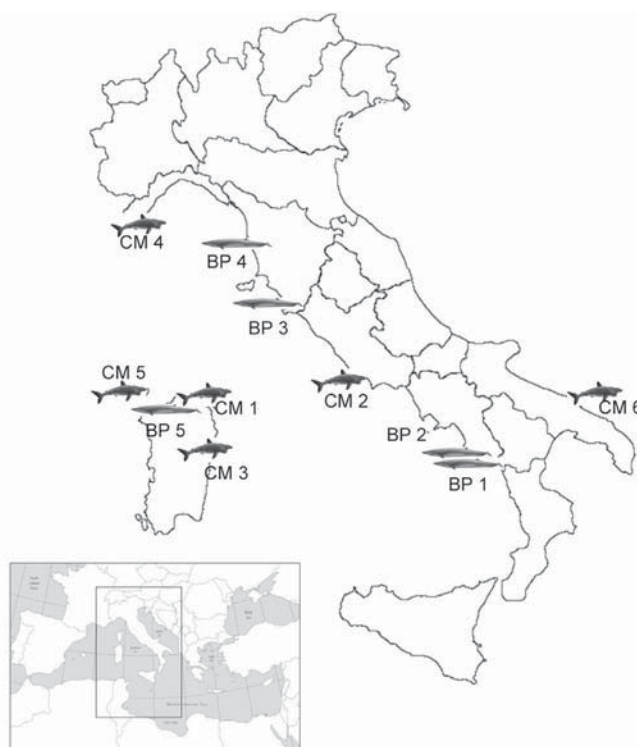


Fig. 1. Details on gender, size, date and location of the stranded specimens of *Balaenoptera physalus* and *Cetorhinus maximus*.

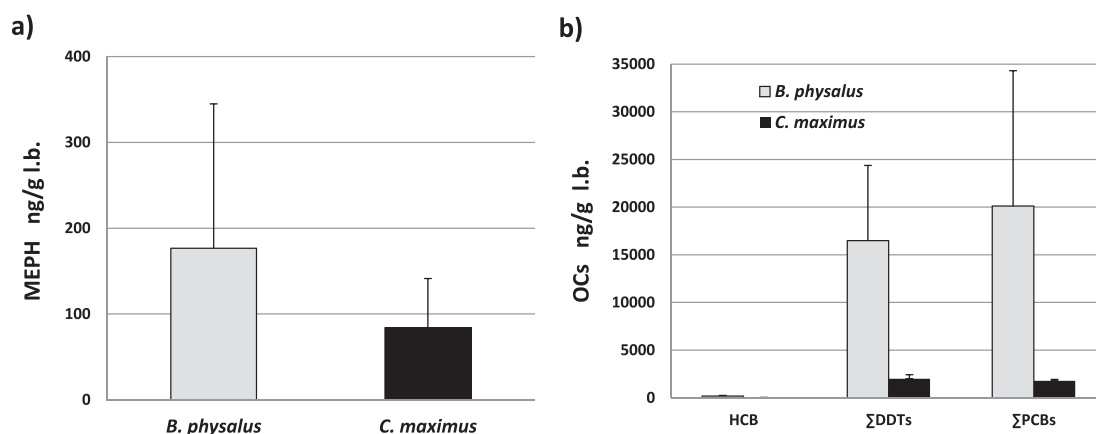


Fig. 2. a) Phthalate (MEHP) and b) Organochlorines concentration (ng/g l.b.) in blubber of Mediterranean *B. physalus* and muscle of *C. maximus*. Bars show mean value \pm standard deviation.

muscle of five stranded fin whales were collected along the Italian coasts during the period 2006–2013 in five different locations. Details on gender and location of the stranded whales are reported in Fig. 1.

2.2. Detection of phthalate content in euphausiids, stranded fin whale and basking shark specimens

DEHP and MEHP were extracted from *E. krohnii* samples (30–40 individuals), plus blubber samples (1 g) and muscle (1 g) of five stranded fin whales and in the muscle (0.5 g) of six accidentally caught basking sharks using the described method. DEHP (di-(2-ethylhexyl) phthalate) and MEHP (mono-(2-ethylhexyl) phthalate) were analyzed from the subsamples following a method described by Takatori et al. (2004) with few modifications. Each sample was thawed, weighted and transferred into a 15 ml tube. To this were added 4 ml of acetone. The sample thus obtained was sonicated for 2 min and stirred for 5 min and then centrifuged for 15 min at 3000 rpm to separate the organic part, containing DEHP and MEHP, from the remainder water. Then, 4 ml of supernatant were placed in a further 15 ml tube. Infranatant was again added to 1 ml of acetone, and was sonicated for 2 min, agitated for 5 min and centrifuged for 15 min at 3000 rpm for a further separation of the organic from aqueous medium. Then the supernatant phase was recovered and rebuilt with that resulting from the first extraction. The supernatants, mixed well, were then evaporated in a centrifugal evaporator. The extract was then resuspended with 0.5 ml of acetonitrile and passed through a nylon filter. Subsequently, the final volume was adjusted to 0.5 ml, which were placed in an autosampler vial and injected into a LC-ESI-MS system. The instrumental analysis was performed using a Finnigan LTQ Thermo LC/MSn 110 with ESI interface. 5 μ l of extracted sample were injected via autosampler in the HPLC system. A reverse phase HPLC column (Wakosil3C18, 2.0 \times 100 mm; Wako Pure Chemical Industries Ltd.) was used. The mobile phases consisted of 100% acetonitrile (A) and 0.05% aqueous acetic acid (B). Elution was performed using an isocratic mode (A/B: 15/85, v/v) at 0.25 ml/min. The chromatographic run for each sample had duration of 30 min. ESI-MS was operated in negative or positive ion mode depending on the analytes (MEHP was detected in negative mode, while DEHP in the positive mode). The heated capillary and voltage were maintained at 500 $^{\circ}$ C and \pm 4.0 kV, respectively. The daughter ions were formed in the collision cell using N₂ gas as the collision gas. The ions used for identification were (parent ion/daughter ion) 277/134,120 and 391/149 for MEHP and DEHP respectively. For the

quantitative analysis four-point calibration curve, prepared by progressive dilution of a solution of the two analytes of interest was used. Blanks were analyzed with each set of five samples as a check for possible laboratory contamination and interferences. Data quality assurance and quality control protocols included also matrix spikes, and continuing calibration verification. The limits of detection (LODs) and limits of quantification (LOQs) for the compounds analyzed are the value of the compound in the blanks +3 SD and +10 SD, respectively; LOD and LOQ were 1 and 2 ng/g respectively for MEHP and 5 and 10 ng/g respectively for DEHP. The analytes levels below the limits of detection (<LOD) were considered with a value equal to the value of the LOD, while, in the cases in which the analyte was present at levels between the LOD and the LOQ, the LOQ value was used.

2.3. Detection of OC concentrations in stranded fin whale and basking shark specimens

Analysis for HCB, DDTs and PCBs were performed according to method of U.S. Environmental Protection Agency (EPA) 8081/8082 with modifications (Marsili and Focardi, 1997). The samples of 1 g of blubber (*B. physalus*) and 1 g muscle (*C. maximus*) were lyophilized in an Edwards freeze drier for 2 days. The sample was extracted with n-hexane in a Whatman cellulose thimble (i.d. 25 mm, e.d. 27 mm, length 100 mm) in the Soxhlet apparatus for 9 h. The sample was spiked with surrogate compound (2,4,6-trichlorobiphenyls – IUPAC number 30, Ballschmiter and Zell, 1980) prior to extraction. This compound was quantified and its recovery calculated. After the extraction, the sample was purified with sulfuric acid to obtain a first lipid sedimentation. The extract then underwent liquid chromatography on a column containing Florisil that had been dried for 1 h in an oven at 110 $^{\circ}$ C. This further purified the apolar phase of lipids that could not be saponified, such as steroids like cholesterol. Decachlorobiphenyl (DCBP – IUPAC number 209) was used as an internal standard, added to each sample extract prior to analysis, and included in the calibration standard, a mixture of specific compounds (Aroclor 1260, HCB and pp'- and op'-DDT, DDD and DDE). The analytical method used was High Resolution Capillary Gas Chromatography with a Agilent 6890N and a 63Ni ECD and an SBP-5 bonded phase capillary column (30 m long, 0.2 mm i.d.). The carrier gas was N₂ with a head pressure of 15.5 psi (splitting ratio 50/1). The scavenger gas was argon/methane (95/5) at 40 ml/min. Oven temperature was 100 $^{\circ}$ C for the first 10 min, after which it was increased to 280 $^{\circ}$ C at 5 $^{\circ}$ C/min. Injector and detector temperatures were 200 $^{\circ}$ C and 280 $^{\circ}$ C

respectively. The extracted organic material (EOM%) from freeze-dried samples was calculated in all samples. Capillary gas-chromatography revealed op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and 30 PCB congeners. Total PCBs were quantified as the sum of all congeners (IUPAC no. 95, 101, 99, 151, 144, 135, 149, 118, 146, 153, 141, 138, 178, 187, 183, 128, 174, 177, 156, 171, 202, 172, 180, 199, 170, 196, 201, 195, 194, 206). Total DDTs were calculated as the sum of op'DDT, pp'DDT, op'DDD, pp'DDD, op'DDE and pp'DDE. The results were expressed in ng/g lipid basis (l.b.). The detection limit was 0.1 ng/kg (ppt) for all the OCs analyzed.

3. Results and discussion

In this study, six muscle samples of accidentally caught basking shark in Italian waters and, plus blubber and muscle samples from in five stranded fin whales (sub-adults and adults) between 2007 and 2012 in five different sites on the Italian coast were analyzed. All these samples were analyzed for phthalates and organochlorines (expressed in l.b.) used as potential tracers of assumption of microplastics during the filtering activities for feeding. Additionally, the crustacean *E. krohnii* was analyzed as one of the major prey of the fin whale and as component of the zooplankton.

The DEHP primary metabolite, MEHP, was analyzed in the stranded specimens and *E. krohnii*. The analysis showed appreciable levels of MEHP in all of the samples, while DEHP was detected in only one sample (data not shown) (Table 1).

Interestingly, concentrations of MEHP are twice as high in the cetacean species compared with the cartilaginous fish (Fig. 2a).

The same trend is shown for the organochlorine concentrations, where for the three classes of OCs investigated (HCB, DDTs and PCBs) were always markedly higher in fin whale specimens compared to basking sharks (Fig. 2b).

Moreover as previously published by Fossi et al. (2012), the presence of harmful chemicals in Mediterranean fin whales, that were hypothesized to be linked with intake of plastic derivatives by water filtering and plankton ingestion, are confirmed by the results of this study, which documents relevant concentrations of MEHP in the blubber of five out of six stranded fin whales. MEHP is a marker for exposure to DEHP, whereas DEHP was never detected in the fin whale samples.

The concentrations of total OCs in the muscle of the three whale specimens are always markedly higher (DDTs mean value: 15,956 ng/g l.b.; PCBs mean value: 16,692 ng/g l.b.) than those found in the muscle of the basking shark (DDTs mean value: 2001 ng/g l.b.; PCBs mean value: 1779 ng/g l.b.). The difference between the two species in the bioaccumulation of fat-soluble contaminants can be linked to a different ability of excretion related to the potential

excretory activity through the gills in fish (Barber, 2008) and bioaccumulation in adipose tissue especially in cetaceans.

The PCBs fingerprint of the two target species was compared with neustonic/planktonic and microplastic samples (NP-MPs) collected in the Pelagos Sanctuary (Fig. 3). Among the 30 PCB congeners analyzed, the highest percentage (43%) is represented by the PCB 195 in the NP-MPs samples that is the second most abundant congener in basking shark, while it was detected in very low percentage in fin whale. This preliminary evidence suggests the use of this PCB congener as tracer of the absorption of POPs through NP-MPs in surface feeding organisms. Moreover, the most abundant congeners in fin whale and basking shark are the PCB 153, a congener also abundant in NP-MPs samples (Fig. 3).

4. Conclusions

The initial insight into microplastic pollution on Mediterranean scale on the concentration levels and spatial distribution of microplastics in the area MPA of Pelagos Sanctuary underline that the mean abundance of microplastics estimated are of the same order of magnitude as that found for the North Pacific Gyre (Moore et al., 2001). This suggests the high occurrence of this emerging threat in the only pelagic MPA of the Mediterranean Sea (Collignon

Table 1

Organochlorine and MEHP concentrations (ng/g l.b.) in the blubber of Mediterranean *B. physalus* (BP) and muscle of *C. maximus* (CM).

| Sample ID | Species | HCB (ng/g l.b.) | \sum DDTs (ng/g l.b.) | \sum PCBs (ng/g l.b.) | MEHP (ng/g l.b.) |
|-----------|--------------------|--------------------|----------------------------|----------------------------|---------------------|
| BP 1 | <i>B. physalus</i> | 129.13 | 6580.67 | 9117.09 | 377.82 ^a |
| BP 2 | <i>B. physalus</i> | 286.26 | 12,284.32 | 8155.00 | 110.68 ^a |
| BP 3 | <i>B. physalus</i> | 157.82 | 21,404.45 | 42,778.45 | 332.31 ^a |
| BP 4 | <i>B. physalus</i> | 180.93 | 26,833.64 | 24,060.13 | 61.06 ^a |
| BP 5 | <i>B. physalus</i> | 201.02 | 15,357.34 | 16,410.87 | 1.48 ^a |
| CM 1 | <i>C. maximus</i> | 41.09 | 1890.42 | 1575.57 | 58.06 |
| CM 2 | <i>C. maximus</i> | 9.52 | 2638.73 | 1710.69 | 113.94 |
| CM 3 | <i>C. maximus</i> | 41.02 | 2177.60 | 1820.77 | 50.39 |
| CM 4 | <i>C. maximus</i> | 10.74 | 1647.66 | 1970.62 | 156.67 |
| CM 5 | <i>C. maximus</i> | — | — | — | 114.37 |
| CM 6 | <i>C. maximus</i> | 21.11 | 1652.64 | 1820.73 | 11.17 |

^a From Fossi et al., 2012.

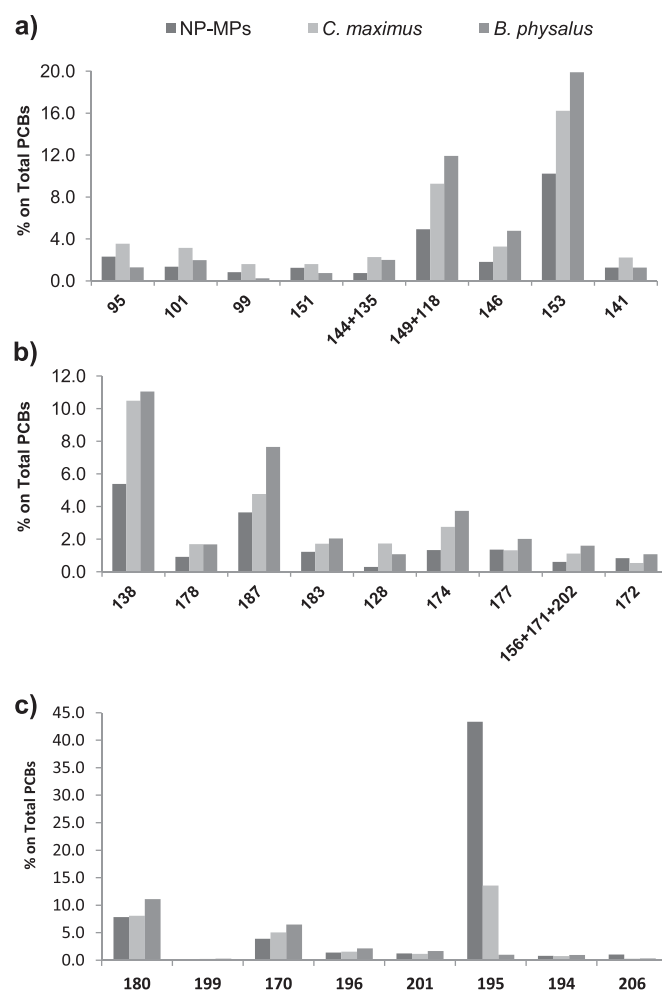


Fig. 3. PCBs fingerprint (30 congeners): bars show the percentage of each congeners calculated on the total concentration of all the congeners analyzed in neustonic/planktonic and microplastic samples (NP-MPs), *C. maximus* and *B. physalus*. Each graph (a–c) shows congeners in the same order as they are revealed by the instrument.

et al., 2012; Fossi et al., 2012). High presence of plastic particles have been detected in superficial neustonic/planktonic from the Pelagos Sanctuary areas investigated (mean value 0.62 items/m³), with levels approximately seven time higher in the samples from the Ligurian Sea (mean value 0.94 items/m³), than the samples compared to the Sardinian Sea (mean value 0.13 items/m³). High concentration of phthalate MEHP and DEHP have been detected, in superficial NP-MPs samples collected in the Pelagos Sanctuary areas (MEHP 53.47 ng/g f.w., DEHP 20.36 ng/g f.w) (Fossi et al., 2012). Moreover, the levels of OCs and microplastic abundance in Mediterranean Sea were recently detected in superficial neustonic/planktonic samples collected in Sardinian sea with PCBs ranging from 1889.6 ng/g d.w. to 3793.1 ng/g d.w. and DDTs from 185.0 ng/g d.w. to 2130.1 ng/g d.w. (de Lucia et al., in this issue).

Until now few studies have addressed the impact of microplastics on filter-feeding organisms or other planktivorous animals (Boerger et al., 2010; Cole et al., 2013; Lusher et al., 2013; Murray and Cowie, 2011; von Moos et al., 2012). A previous study by Fossi et al. (2012) has reported on the potential impact on large filter-feeding organism such as baleen whales.

In the present paper, we explore the potential routes of exposure and or absorption of MPs in the Mediterranean fin whale and basking shark in relation to their different filter feeding activities (Table 2).

Basking sharks can sieve small organisms and microdebris from the water. Swimming with mouth open, masses of water fill the basking shark mouth with prey flow. After closing its mouth, the shark uses gill rakers that filter the nourishment from the water. Gill rakers have thousands of bristles in the shark's mouth that trap the small organisms and microdebris which the shark then swallows. The water is expelled through the shark's pairs of gill slits. The seawater filtration rate for a 7 m basking shark (mouth gape area ca. 0.4 m²) swimming at a speed of 0.85 m s⁻¹ was calculated to be 881 m³ h⁻¹; we can hypothesize that in the Pelagos Sanctuary areas (mean MPs value 0.62 items/m³), this species could consume approximately 540 MPs items h⁻¹, for a total daily consumption of approximately 13,110 microdebris items, plus any related adherent or incorporated toxic chemicals such as OCs, PAHs and phthalates (Table 2).

Fin whales exhibit one of the most extreme feeding methods among aquatic vertebrates. Fin whales, and other *Balaenopteridae*, lunge with their mouth fully agape, thereby generating dynamic pressure to stretch their mouth around a large volume of prey-laden water, which is then filtered by racks of baleen (Goldbogen et al., 2007). *Balaenopteridae* are intermittent filter feeders that ingest mouthfuls of water and separate food from the water before expelling it and, subsequently, swallowing the prey captured. The filtering apparatus of baleen whales can be compared to a net or a sieve, depending on the prey, microdebris and water condition through baleen fringes (Werth, 2001). Considering the seawater filtration rate approximately of 5893 m³ daily we can hypothesize

Table 2
Comparison between total volume filter daily, total plankton consume daily and theoretical number of MP items assumed by *B. physalus* and *C. maximus*.

| | <i>Balaenoptera physalus</i> | <i>Cetorhinus maximus</i> |
|--|------------------------------|------------------------------------|
| Average adult body length | 20 m | 7 m |
| Average adult body mass | 50,000 kg | 4000 kg |
| Engulfment volume | 71 m ³ | – |
| Filtration rate | – | 881 m ³ h ⁻¹ |
| Number of lunges day ⁻¹ | 83 | – |
| Total volume filtered daily | 5893 m ³ | 21,144 m ³ |
| Total plankton consumed daily | 913 kg | 30.7 kg |
| Theoretical number of MPs items assume daily | 3653 | 13,110 |

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that fin whale surface feeding in the Pelagos Sanctuary areas (mean MPs value 0.62 items/m³) could consume, a total daily amount of approximately 3653 items and the relative sink toxic chemicals (Table 2). Experiment carried out on porosity of baleens in whales using polymer microsphere (mean particle size 710 μm) pointed out that suspended particles did not remain on baleen fringes and prey and items fall onto the tongue upon water expulsion (Werth, 2013). This mechanism suggests that microdebris can be ingested by the whale together with the prey. Considering this theoretical calculation, the basking shark can ingest daily approximately a total intake of 3.6 time more MP items than the fin whale. Although, this higher intake of MPs is however coupled to values of phthalates two time lower and of OCs three times lower than those found in fin whale. The marked difference between the two species in the bioaccumulation of phthalates and organochlorines can be linked both to a different ability of excretion of contaminants related to the presence of a high excretory activity through the gills in the basking shark but also to the massive ingestion of euphausiid species by fin whale (total plankton consume daily 913 kg) that show high concentrations of plastic additives (Fossi et al., 2012). It is well known that the fin whale in the Mediterranean Sea feeds preferentially on the planktonic euphausiid *Meganyctiphanes norvegica*, even if it feeds on a wide spectrum of marine organisms, ranging from copepods to other euphausiid species, to small schooling fish (like *Thysanoessa inermis*, *Calanus finmarchicus*, *E. krohnii*) (Notarbartolo di Sciara et al., 2003; Relini et al., 1992). Preliminary data on MEHP concentration in samples of *E. krohnii* collected in Sicilian Channel show high concentration of this contaminant, ranging from 8.35 to 51.14 ng/g (mean values 36.92 ng/g) and suggesting the presence of plastic additives also in planktonic species living in the water column. Evidences of ingestion and impact of MPs by invertebrates, in particular zooplankton, have been reported (Cole et al., 2013; Murray and Cowie, 2011). Beside the physical harm and toxicological risk for invertebrates and zooplanktonic species themselves caused by MPs and through feeding activity, the trophic transfer across the food chain represent a serious concern, especially for planktivorous species such as baleen whales and basking sharks.

Considering both the high presence of MPs in the Mediterranean environment, and particularly in the MPA of Pelagos Sanctuary, and the detection of plastic additives and OCs in the tissues of basking sharks and fin whale, large filter feeding marine organisms appear to be chronically exposed to persistent and emerging contaminants related to prey and MPs ingestion. Rochman et al. (2014) underline that several classes of compounds can be carried and released by MPs since organisms living in high density MPs environment show higher plastic-derived chemical pollutants accumulation in their tissue. The dual sources of contamination could derive from direct leaching of contaminants (sorbed on or additive) from microplastics and assumption through already contaminated plankton prey.

In this context, the data in this paper suggest the use of phthalates as a tracer of microplastic ingestion by fin whale and basking sharks. The tracer can serve as a warning signal of exposure to endocrine disruptors such as MEHP in the endangered Mediterranean population of this baleen whale and cartilaginous fish.

Particular attention has also been given to this new field of research during the recent workshop organized by IWC and Woods Hole Oceanographic Institution in May 2013 in Woods Hole (MA, USA) on Assessing the Impacts of Marine Debris on Cetaceans. The workshop recommended that baleen whales and other large filter feeders should be considered as critical indicators of the presence and impact of microplastics in the marine environment, in national and international marine debris strategies. The workshop

encouraged also further non-lethal research and the biomarker development on these endangered Mediterranean species (IWC, 2013).

The present study represents the first evidence of plastic additives (phthalates) in Mediterranean basking sharks and it underlines the importance of future research both on detecting the presence of and looking for toxicological impacts of microplastics in filter-feeders species such as cetaceans mysticete, basking shark and devil ray. Due to the wide home-range and high-mobility of these species, which move in the whole basin all year round, they could represent a wide scale integrator of the ecotoxicological status of the entire Mediterranean basin. Moreover, occupying these species the lowest position of the food web can be considered as an early warning of the presence of a mixture of contaminants in the marine food chain.

We highlight the value of these species in the implementation of the Descriptor 8 (contaminants) and Descriptor 10 (marine litter) in the European MSFD, as sentinels of the plastic-related contamination and presence and impact of micro-litter in the pelagic environment.

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Monitoring the impact of litter in large vertebrates in the Mediterranean Sea within the European Marine Strategy Framework Directive (MSFD): Constraints, specificities and recommendations

F. Galgani^{a,*}, F. Claro^b, M. Depledge^c, C. Fossi^d

^a Ifremer, Immeuble Agostini, ZI Furiani, 20600 Bastia, Corsica, France

^b Muséum national d'Histoire naturelle, GTMF, CP41, 57 rue Cuvier, 75231 Paris cedex 05, France

^c University of Exeter, Devon EX4 4QJ, United Kingdom

^d University of Siena, Via Mattioli 4, 53100 Siena, Italy

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ABSTRACT

In its decision (2010/477/EU) relating to the European Marine Strategy Framework Directive (MSFD, 2008/56/EC), the European Commission identified the following points as focuses for monitoring:

(i) 10.1.1: Trends in the amount, source and composition of litter washed ashore and/or deposited on coastlines,

(ii) 10.1.2: Trends in the amount and composition of litter in the water column and accumulation on the sea floor,

(iii) 10.1.3: Trends in the amount, distribution and composition of micro-particles (mainly micro-plastics), and

(iv) 10.2.1: Trends in the amount and composition of litter ingested by marine animals.

Monitoring the impacts of litter will be considered further in 2014. At that time, the strategy will be discussed in the context of the Mediterranean Sea, providing information on constraints, protocols, existing harm and research needed to support monitoring efforts.

The definition of targets and acceptable levels of harm must take all factors into account, whether entanglement, ingestion, the transport and release of pollutants, the transport of alien species and socio-economic impacts. It must also reflect on the practical deployment of “ingestion” measures (10.2.1). The analysis of existing data will reveal the potential and suitability of some higher trophic level organisms (fish, turtles, birds and mammals) for monitoring the adverse effects of litter. Sea turtles appear to be useful indicator species, but the definition of an ecological quality objective is still needed, as well as research on alternative potential indicator species.

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1. Introduction

The Mediterranean Sea is the planet's most highly-affected area in terms of marine litter, with densities of over 100,000 items/ha found on the sea floor, together with microplastics at 892,000 items/km² (Barnes et al., 2009; Collignon et al., 2012). In recent years, marine litter has caused increasing harm due to ingestion, entanglement and the transport of toxic or living organisms (Gregory, 2009).

In order to coordinate the protection of the marine environment, the European Commission has developed the Marine

Strategy Framework Directive (MSFD) as part of its “Integrated Maritime Policy” (Markus et al., 2011). This directive establishes a framework for each Member State to take action to achieve or maintain Good Environmental Status for the marine environment by 2020. Member States are obliged to follow an “action plan”, including an initial assessment of current environmental status, determine what Good Environmental Status (GES) is, then establish a series of environmental targets and associated indicators.

The creation and implementation of a monitoring programme for ongoing assessment and regular target updating was launched recently. A series of measures designed to achieve or maintain good environmental status will complete the process by 2016, with a follow-up assessment scheduled in 2018.

Among the 11 descriptors incorporating 56 indicators of Good Environmental Status, descriptor 10 is identified as “Properties and

* Corresponding author.

E-mail address: francois.galgani@ifremer.fr (F. Galgani).

quantities of marine litter do not cause harm to the coastal and marine environment". "Litter" refers to items that have been made or used by people, then deliberately discarded or lost in the sea and on beaches, including material transported into the marine environment from land by rivers, drainage or sewage systems, or winds. Much of this litter will persist in the sea for years, decades or even centuries. On average, three quarters of all marine litter consists of plastics, which are known to be particularly persistent (Galgani et al., 2010).

The Commission's Decision (2010/477/EU) of September 1st, 2010 on "Criteria and methodological standards on Good Environmental Status of marine waters" identifies the following four indicators for monitoring progress towards achieving Descriptor 10:

(i) Trends in the amount of litter washed ashore and/or deposited on coastlines, including analysis of its composition, spatial distribution and, where possible, source (10.1.1), (ii) Trends in the amount of litter in the water column (including floating at the surface) and deposited on the sea floor, including analysis of its composition, spatial distribution and, where possible, source (10.1.2), (iii) Trends in the amount, distribution and, where possible, composition of microparticles (in particular microplastics) (10.1.3), and (iv) Trends in the amount and composition of litter ingested by marine animals (e.g. stomach analysis) (10.2.1) for the evaluation of its impact on marine fauna organisms.

These Indicators were drawn up by a group of specialists on the basis of an assessment of what could be defined as Good Environmental Status (Galgani et al., 2010). However, knowledge relating to the distribution of litter, its degradation and fate, and its potentially harmful biological, physical and chemical impacts on marine life and habitats is currently insufficient.

Various monitoring programmes are to be implemented by 2014. The definition of targets and acceptable levels of harm will mainly rely on the practical implementation of the "ingestion" indicators (10.2.1) listed in the MSFD. The strategy has been well-defined for indicators of adverse impacts in the Mediterranean Sea, but its limitations must be identified and its scientific basis must be made more robust. UNEP pointed out that the main legal and institutional frameworks affecting the Mediterranean on this topic underline a general lack of available data on marine wildlife affected by marine litter in the Mediterranean Sea.

Marine litter has a major impact on large vertebrates, as animals often become entangled in discarded ropes and nets, or trapped in plastic containers and strapping bands. Many animals also mistake litter items as prey: up to 76% of turtles have been recorded as ingesting plastic bags in certain Mediterranean areas (Tomas et al., 2002), while the stomachs of 98% of Fulmars in the North Sea have been found to contain plastic, potentially leading to a loss of physical condition, breeding failure and, in severe cases, to death (Van Franeker et al., 2011). Mediterranean seals and other marine mammal species are subject to entanglement, but large vertebrates may also be exposed to micro-litter ingestion through passive ingestion, in particular filter feeding (Fossi et al., 2012a).

The analysis of existing data reveals the potential and suitability of some higher trophic level organisms as indicators for monitoring the effects of litter (Fig. 1). Here, we describe the opportunities and limitations of using large vertebrates to evaluate the harm caused to marine life by litter. We also highlight several key issues that must be addressed through research. Finally, various recommendations are given to support the implementation of monitoring programs.

2. MSFD and the monitoring of marine litter

In Article 11 of the monitoring requirements of the MSFD, it is specified that Member States must establish and implement

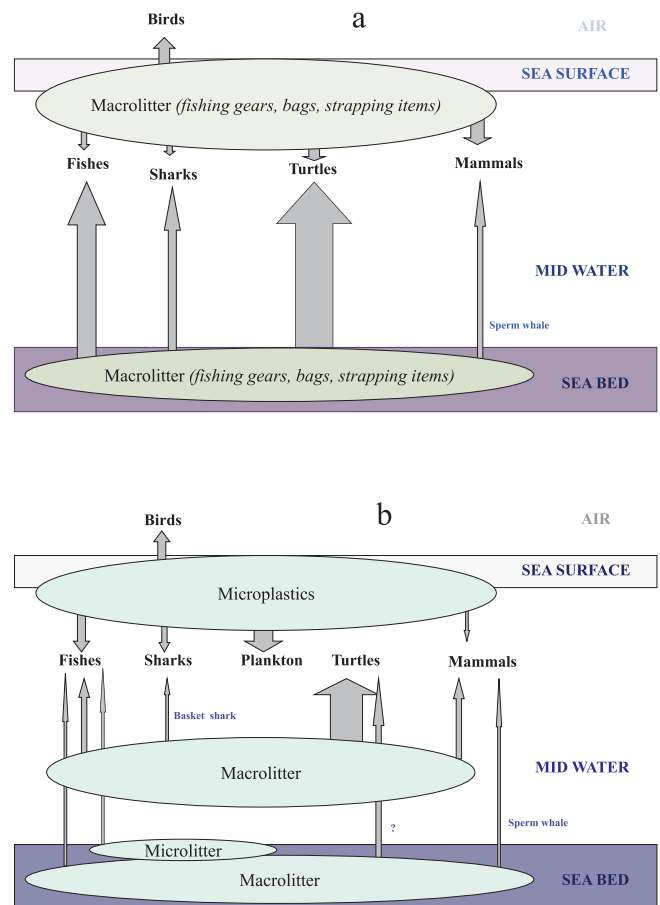


Fig. 1. Summary of interactions between large marine vertebrates and marine litter. Fluxes of litter in the life cycle and intensity of its effects on large marine vertebrates, (a: entanglement; b: ingestion), depending on various factors such as ingestion mechanisms (predation, active or passive filter feeding), development stage (benthic or pelagic phases for sea turtles), behaviour and foraging strategy (feeding on the sea floor, in the water column or on the surface, selectivity according to colour, shape etc, ecological plasticity in diet and habitat), types of litter (micro/macro litter) and types of fishing gear (nets, hooks and lines). The thicker arrows indicate key processes. Although trophic transfer from one level to another has been demonstrated in vitro for microplastics in plankton, it remains controversial in situ, as most ingested litter is excreted in faeces. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coordinated monitoring programmes for the ongoing assessment of the environmental status of their marine waters and that monitoring programmes within given marine regions or sub-regions must be compatible.

The monitoring efforts will address various objectives, such as the assessment of GES, temporal and spatial trends, the level of achievement of environmental targets and the effectiveness of measures. Monitoring will mainly consist of determining trends in the amounts of marine litter and its adverse impacts, and the identification of sources, in order to support measures to reduce plastic inputs. Indicators that are to be directly included in national monitoring plans in 2014 and indicators requiring further development will typically be considered separately.

In the context of the Mediterranean sea and Barcelona Convention, a Policy Document and associated Strategic Framework for Marine Litter management was adopted through MEDPOL in 2012 to (i) follow trends of marine litter generation and distribution through the establishment of a monitoring programme for marine litter based on the Ecosystem Approach, (ii) indicate sources and activities leading to marine litter production, and (iii)

indicate if the adopted litter management/mitigation strategies are effective or need further adaptation.

A litter monitoring programme is expected to be developed for the application of the Ecosystem Approach at a regional level, with MEDPOL coordinating this activity and promoting the appropriate methodologies. The Regional Action Plan (RAP) on marine litter, adopted in December 2013, shall become legally binding once it has been adopted by the Contracting Parties of the Barcelona Convention. Article 12 of the RAP refers to a Mediterranean Marine Litter Monitoring Programme for 2014, following international and regional guidelines adapted from the protocols produced by the MSFD technical group on marine litter (Galgani et al., 2011; Galgani et al., 2014). This technical group tackles the comparison and final assessment of the various existing monitoring methods, as well as other crucial issues that must be addressed and clarified (spatial distribution of survey sites, sampling frequency, QA/QC needs, management/handling of data/metadata, etc.).

The impacts of nano and microplastics at sea span many biological levels, from molecular to ecosystem. They include chemical and mechanical impacts due to ingestion, the release of transported chemicals and the transport of alien species (Fossi et al., 2012a; Cole et al., 2013; Rochman et al., 2013). Entanglement by, and ingestion of, larger debris generally affect large organisms, whether on an individual or population level. However, larger debris may also affect smaller organisms and ecosystems through transportation to new, remote habitats, albeit to a lesser extent.

Biota indicators play an important role, as they provide indications of possible harm. At the same time, current protocols and methods have varying degrees of maturity. Pilot-scale monitoring is therefore an important step towards monitoring litter harm in terms of determining baselines and/or adapting the strategy to local areas. Regarding harm assessment in the Mediterranean sea, and in accordance with Article 11 of the MSFD, Member States sharing a marine region or sub-region must also draw up their monitoring programmes in a coherent manner by ensuring monitoring methods are consistent across the marine region or sub-region; this will facilitate the comparison of monitoring results and take into account relevant trans-boundary impacts and features. Without some degree of information on trends and amounts across all compartments, a risk-based approach to litter monitoring and measures is impossible.

When defining the aims and objectives of monitoring, the Marine Strategy Coordination Group (MSCG) interpreted monitoring as an assessment of whether GES has been achieved or maintained, whether environmental status is improving, stable or deteriorating, and what progress has been made towards achieving environmental targets.

Our current lack of knowledge with regards to harmful levels of litter is such that absolute targets are difficult to set; as a result, many Member States are formulating trend targets instead. The protocol for litter ingested by Fulmars (Van Franeker and the SNS Fulmar Study Group, 2011; Van Franeker et al., 2011) is an exception: a quantitative target level has been formulated as an OSPAR Ecological Quality Objective (EcoQO) and has recently become a legal obligation in certain member states. The design of most protocols enables regional adaption and the discrimination of litter items; they are therefore likely to detect changes in litter types and enable a proper assessment of the various measures implemented. For example, monitoring of litter in Fulmar stomachs in the OSPAR area has shown decreasing trends in industrial plastic pellets after measures were taken to decrease pellet spillage (Galgani et al., 2011). As major future decisions will be based on measures, monitoring efforts should be shouldered by quality control/quality assurance (inter-comparisons, use of reference material, training, etc.) to assist survey teams. A master-standard list of categories of

litter items has been prepared by the MSCG Technical Group ML with compatible sub-categories for the various marine compartments (beach, sea floor, sea surface and ingested litter). This will enable the comparison of results between regions and environmental compartments. Items may be attributed to a given source e.g. fisheries, shipping etc., or a given form of interaction e.g. entanglement, ingestion etc., hence facilitating identification of the main sources of marine litter pollution and the potential harm caused by litter. This will enable a more target-orientated implementation of measures.

Site selection strategies will focus on both sites with specific characteristics and sites chosen randomly in order to facilitate extrapolations. Regarding harm indicators in larger organisms from the Mediterranean sea, the implementation of sampling campaigns on species (turtles, fish, mammals or birds) that are widely distributed and may migrate over long distances, while taking into account the characteristics of the sampling area (generally on a basin/subbasin level), will assist in creating a large-scale monitoring network catering for transboundary issues, despite the difficulties and costs involved in finding statistically-significant trends. Moreover sampling/analysis will need to be coordinated and harmonized on a basin scale, e.g. Northwestern Mediterranean, Adriatic, Ionian, Aegean and Levantine basins.

Our ability to unveil significant trends or variations may be assisted by case-specific statistical power analysis. This has been conducted for various protocols, e.g. the Fulmar litter ingestion protocol, and increases the probability of detecting actual changes.

Data handling and reporting for the MSFD are under consideration both at an EU level and a Regional Sea and sub-regional level. An online, European-wide data collection system will facilitate analysis. The reporting process for data and information under the MSFD (Art 19.3) is being addressed by the DIKE Working Group (Data, Information Knowledge Transfer) and steered by DG Environment and the European Environmental Agency, to define data that will be collected at a national level and made available through INSPIRE or EMODnet in the framework of the Water Information System for Europe (WISE–Marine).

3. Adverse impacts and monitoring

The potentially harmful implications of marine litter have escalated in recent years and currently include: (i) harm to and mortality of marine wildlife as a consequence of the ingestion of marine litter, (ii) entanglement in derelict nets, traps and pots leading to potential losses in biodiversity, (iii) the accumulation and transport of persistent organic pollutants and the release of potentially toxic and hormonal-effective chemicals, and (iv) transport of alien species (Gregory, 2009).

Defining harm in the MSFD context is a difficult task. A variety of approaches or guidelines can be used to evaluate the adverse effects of litter at sea. Reference documents such as the UK government's Farm Animal Welfare Council, which takes into account 5 types of effects, named freedoms, are useful (FAWC, 1979). However, we do not possess a complete picture of the effects of marine litter. Moreover, its economic impacts, as referred to in the GES definition, are not taken into consideration in this approach.

Litter affects marine life at various organisational levels and its impact varies according to the target species or population, environmental conditions and the considered region or country. The concept of harm itself is not obvious, as no acceptable units of measure have been defined. A reassessment of what Good Environmental Status means will help better define harm within MSFD. However, even proven harm may not be useful for monitoring purposes. In view of the diversity of litter and targets,

measurements may prove unsuitable for the large-scale, complex harmonisation currently necessary.

For example, entanglement has been highlighted as having one of the most harmful impacts on marine organisms, with culprits such as strapping bands, plastic bags and drums. As a result of their size and weight, larger vertebrates may continue to travel over considerable distances after becoming entangled in ropes, net and lines, hence transforming active fishing gear into marine debris. Reports also exist of small cetaceans, such as harbour porpoises, becoming entangled in ghost fishing gear (Butterworth et al., 2012). However, the MSFD monitoring criteria only refer to ingested litter, due to difficulties in distinguishing between entanglement in litter and active fishing gear. The European situation with regards to animal deaths due to entanglement is somewhat diverse. In certain areas, with efficient stranding networks, entangled beached species such as cetaceans are quite frequently found and could therefore contribute to harm assessment. However, current difficulties in interpreting data, together with the low reported numbers of entangled beached animals and problems associated with large-scale harm assessment due to the rarity of strandings, mean this approach can only usefully be applied to specific areas and on the basis of national decisions. Nevertheless, research may contribute to the development of new, more specific entanglement indicators. For example, seabird nests can be used to facilitate litter-related entanglement monitoring, as the litter found there cannot originate from active fishing gear (Votier et al., 2011). Optimising this type of protocol should promote a better understanding of harmful effects and more efficient MSFD implementation in the future.

An emerging area of concern is the accumulation of microplastic fragments (less than 5 mm) in the water column and in sediment, which also affect marine life and especially filter feeders (Thompson et al., 2004).

Several recent studies have identified the potential effects of plastic particles, in particular on invertebrates and fish, including: 1 – transport of persistent, bioaccumulating and toxic (PBT) substances from plastics. 2 – leaching of additives from the plastics such as phthalates. 3 – physical harm (Wright et al., 2013).

However, until the recent work of Fossi et al. (2012a,b), no data had been reported on the impact of microplastics on large filter-feeding organisms such as baleen whales or sharks, which potentially ingest micro-litter while filter feeding.

As no single species can actually provide full coverage of all of Europe's maritime zones, a range of species is needed to monitor ingested litter, possibly with some spatial overlaps. An indicator expressing the impact of marine litter is available in the North Sea (OSPAR EcoQO); it is used to measure ingested litter in the Northern Fulmar (*Fulmarus glacialis*) with regards to a set target for acceptable ecological quality in the North Sea (Van Franeker and the SNS Fulmar Study Group, 2011, Van Franeker et al., 2011). This tool is applicable to most Northeast Atlantic countries, but research (proportions of micro and macro debris found in stomachs, particles sizes, etc.) is needed, especially for the Mediterranean Sea, and further afield if possible. On the basis of available information and expertise, the MSCG TG ML group reported on the development of monitoring protocols for other species of seabirds, sea turtles and fish. A similar approach to the Fulmar approach has been suggested, whereby various plastic categories are counted and weighed.

A protocol for sea turtle monitoring, focussing on various parameters, is currently being developed for application in the Mediterranean Sea and some areas of the South Atlantic Sea. As post-mortem examinations reveal litter in 30–80% of endangered Loggerhead sea turtles *Caretta caretta* (IUCN red list status "Endangered A1abd ver 2.3", IUCN, 2013 and species listed in CITES Appendix I, review in Schuyler et al., 2013) stranded on beaches or accidentally captured in the northern area of the Western

Mediterranean (Claro and Hubert, 2011; Pibot and Claro, 2011), on the western coast of Italy (Travaglini et al., 42%, $N = 60$ & Camedà et al., 30%, $N = 30$, this issue), in the Adriatic Sea (Lazar and Gracan, 2011), and on the coasts of Tuscany (Campani et al., 2013) and Spain (Tomas et al., 2002), this species is a good candidate for monitoring the Mediterranean on a sub-basin scale (Northwestern Mediterranean, Adriatic, Ionian, Aegean and Levantine basins). This protocol will need to be further improved through (i) more efficient networks dedicated to the collection of stranded animals and measurement of the impact of fishing gear, (ii) the collection of information from rescue centres, (iii) special consideration of ML information from existing monitoring efforts, (iv) the consideration of new findings and (v) monitoring the effects of litter in living organisms using a non-destructive biomarker approach. A pilot study evaluating methods and potential sources of bias was conducted in 2012 in Italy (Matiddi et al., 2011). Research must now be performed to define a Sea Turtle EcoQO suitable for the various areas in the context of the MSFD. This may also constitute an objective for Southern OSPAR regions (France, Spain and Portugal).

The overall threat incurred by ingested macroplastics and other debris remains unclear with regards to all populations and cetacean species. Debris ingestion in 43 cetacean species has been reported in the literature, comprising 7 Mysticete and 35 Odontocete species (Baulch and Perry, 2012; International Whaling Commission, 2013). However, if we look at research on the ingestion of debris by marine mammals, even in view of the spectacular cases of mortality due to ingestion of large amounts of marine litter in the Mediterranean Sea (Jacobsen et al., 2010; De Stephanis et al., 2013), the known rates of incidence of ingested litter are generally too low to justify a standard MSFD monitoring recommendation. For example, less than 1% of mammals stranded on French beaches between 1972 and 2006 were found to have ingested litter (Pibot and Claro, 2011). However, we do have an adequate understanding of variations in species distribution and behaviour at different times of the year, which can be extremely valuable integrators of environmental quality. Discussions at the SETAC workshop in 2013 (Fossi et al., 2012c) revealed that large pelagic fish may be especially useful for monitoring short to medium-term changes in pelagic ecosystems, while marine mammals such as whales provided a more integrated view in the long term (Fossi et al., 2012a).

Although these are early days for suggesting a particular protocol and assessing ongoing research activity, the MSFD marine litter TSG identified the need to develop a common protocol for the monitoring of ingested litter in fish, applicable at all sites, as a priority. Information is still required on the size/types of items to be examined and fish species that are suitable from an ecosystem perspective and for regional comparison (abundance, cycles, etc.). Regular fish monitoring campaigns are already conducted in the Mediterranean Sea (<http://www.sibm.it/SITO%20MEDITS/> and pelagic fish surveys), involving shared sampling efforts and using tried and tested protocols derived from methods for analysing the content of fish stomachs (Cortes, 1997).

Plastic ingestion by seabirds has been widely recorded, being Procellariiformes the most affected order (Rodríguez et al., 2012). Despite the voluminous information on ingested plastic debris by adults across the world (Van Franeker and the SNS Fulmar Study Group, 2011; Van Franeker et al., 2011; Rodríguez et al., 2012), only one study described however plastic ingestion in Mediterranean seabirds (Codina-García et al., 2013). With occurrences of debris in 70–94% of individuals, when the rest of species were below 33% the results pointed out the threatened shearwater species as being particularly exposed to plastic accumulation. Implementation of monitoring may be however limited because of the distribution of these three endemic species, restricted to the western Mediterranean sea.

If abundance of ingested debris by seals has been mentioned as a potential indicator of marine litter in the European Marine Strategy Framework Directive, a recent study (Bravo Rebolledo et al., 2013) demonstrated however an incidence on 11% of the harbour seal from the Dutch coast. In the Mediterranean sea where fewer than 600 individuals of *Monachus monachus* are remaining, no study has been performed but if observed level of incidence may be of environmental concern, the distribution is patchy and the abundance is low in the sense of suitability for monitoring purposes.

In addition to ingestion protocols, guidelines are currently being developed for litter in seabird nest structures and the associated entanglement in litter in nest structures. Some species tend to incorporate marine litter in their nests, which may result in entanglement (Votier et al., 2011). Although regional occurrence and distribution vary, nesting material and associated mortalities can be linked to the amounts of litter found in the natural environment in the vicinity of the breeding site, hence demonstrating the harmful effects of litter with more ease versus litter ingestion. Monitoring can continue to focus on existing colonies that are regularly monitored in many European countries without too much extra effort, but research is still needed to define behaviours, breeding seasons and the types of litter brought into seabird nests, in particular any litter that may originate from land. Information is grossly lacking on Mediterranean nesting species such as European shags (*Phalacrocorax aristotelis*), but the potential remains important, especially with regards to monitoring of remote islands.

The European Shag is very common throughout the Mediterranean and nests on coastal areas in most European and North African countries, together with the Black Sea coast. Litter in shags' nests is already used as indicator of marine pollution in Brittany, France (Cadiou et al., 2011) and the protocol may serve as a basis for implementing the MSFD.

Only a limited number of reports exist on microparticle sampling in biota. Most of these relate to small organisms. Among the larger vertebrates, baleen whales and, potentially, basking sharks are exposed to micro-litter ingestion as a result of their filter-feeding activity, but impacts are under investigation (Fossi et al., 2012b). These large, filtering marine organisms were recently selected for the MED-SDSN PLASTIC-BUSTERS project (under the UN umbrella) as wide-scale indicators of the presence and impact of microplastics throughout the Mediterranean pelagic environment. The fin whale (*Balaenoptera physalus*), which is one of the largest filter feeders in the world, feeds primarily on planktonic euphausiid species. This whale, which is the only resident mysticete in the Mediterranean Sea, gathers in the feeding grounds of the Pelagos Sanctuary Marine Protected Area (MPA). With each mouthful, the whales trap approximately 70,000 l of water, and their feeding activities include surface feeding. The basking shark (*Cetorhinus maximus*) is a large, filter-feeding, migratory and widely-distributed pelagic species. Basking sharks feed on zooplankton captured by forward swimming with an open mouth, so that a passive water flow passes across the gillraker apparatus. Both species could face risks due to the ingestion and degradation of microplastics. The recent workshop (May 2013) organized by the International Whaling Commission (IWC) at the Woods Hole Oceanographic Institution recommended that baleen whales and other large filter feeders be considered in national and international marine debris strategies (e.g. Descriptor 10 (marine litter) in the EU Marine Strategy Framework Directive) as critical indicators of the presence and impact of microplastics in the marine environment. As this is the only example of microplastic ingestion by large vertebrates and the first warning of this emerging threat to baleen whales, and despite the difficulties of sampling, this approach must be considered for further investigation.

Plasticizers, sealing agents and additives such as phthalates or bisphenol A have been shown to be present in the tissues of various marine organisms, including large vertebrates such as whales and sharks (Fossi et al., 2012b,c). Their presence in tissues such as blubber from stranded fin whales (*Balaenoptera physalis*) or muscle from basking sharks (*C. maximus*) suggests that they could serve as a tracer of microplastic intake.

However, although these plastic derivatives have major potential toxicity, their monitoring in the MSFD framework is typically associated with Descriptor 8.

4. Research

In addition to the identification of sources and monitoring-based support, a better understanding of the harm to large marine vertebrates caused by marine litter is needed to support Member States in the implementation of Descriptor 10. However, there is no consolidated common understanding of what constitutes “harm” from marine litter, or how it can be assessed. Research efforts to develop robust approaches for harm assessment will need to be identified. The identification of gaps in our understanding and the development of research programmes are key prerequisites, so that monitoring tools and protocols can be implemented realistically and cost-effectively.

The recent workshop (May 2013) organized by IWC also encouraged further non-lethal research in view of the promising research on biomarker development. It recommended further work in this field, such as new gene expression biomarkers in cetaceans developed in an “ex vivo” approach (organotypic cultures), which exposes cetacean skin biopsies to increasing doses of contaminant mixtures.

Many European projects got underway in 2012/2013. Some have now been completed, but most are still in progress with projected results in 2014–2015. The EC is currently defining the research programmes for Horizon 2020, in which MSFD marine litter research must be taken into account. Priority should be given to the most-affected areas, the most prominent of which is probably the Mediterranean Sea. As stated by the MSCG Technical Group regarding impacts and ingestion, monitoring implementation and short-term research priorities will need to (i) Develop or use existing comprehensive models to define source and destination regions of litter (especially accumulation areas), (ii) Evaluate the environmental consequences of litter-related chemicals (Phthalates, bisphenol A, etc.) in marine organisms using specific diagnostic biomarkers, (iii) Establish the environmental consequences of micro-litter to establish potential physical and chemical impacts on wildlife, (iv) Evaluate the effects of litter on metabolism, physiology, survival rate, reproductive performance and, ultimately, on populations or communities, (v) Study dose/response relationships in relation to types and quantities of marine litter, to enable science-based definitions of threshold levels, and (vi) Rationalise monitoring (standards/baselines; data management/quality assurance; extend monitoring protocols to all Mediterranean sub-regions).

The development and implementation of assessment and monitoring campaigns, and the implementation of measures in the framework of the Mediterranean Action Plan, will also require scientific cooperation among the Parties involved. The Secretariat of the Barcelona convention will organize and support this scientific cooperation, advise the EU Project in terms of scientific needs and support action on priority research topics. In view of the common concerns of the MSCG TG with regards to harm monitoring, research priority will be given to (i) The development of comprehensive modelling tools for the evaluation and identification of sources and fate of litter in the marine environment, (ii)

Table 1
Recommendations for monitoring and research litter ingestion by large marine organisms within MSFD.

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|---|
| <p>A) Monitoring</p> <p><i>Turtles (high priority):</i></p> <ul style="list-style-type: none"> - Define an EcoQO for the loggerhead turtle <i>Caretta caretta</i> in the Mediterranean Sea (High priority) - Set up harmonised monitoring at a sub-regional level (basin level) - Improve the collection of information/samples (stranded turtles and from fishermen) <p><i>Birds:</i></p> <ul style="list-style-type: none"> - Evaluate the potential value of nested litter as an indicator for the evaluation of GES and as a monitoring tool <p><i>Fish:</i></p> <ul style="list-style-type: none"> - Evaluate the potential value of fish for monitoring the ingestion of litter by marine organisms <p>B) Research</p> <p><i>Birds:</i></p> <ul style="list-style-type: none"> - Identify bird species suitable for the development of a Fulmar type EcoQo - Improve understanding of the impact of litter on nesting birds <p><i>Turtles:</i></p> <ul style="list-style-type: none"> - Improve understanding of turtle migration in the Mediterranean Sea. - Improve understanding of how litter is affecting organisms (digestion, physiology, reproduction, population dynamics, etc.) <p><i>Mammals and sharks:</i></p> <ul style="list-style-type: none"> - Understand interactions between long term marine environmental changes and litter effects on mammals for the assessment of the quality of pelagic marine ecosystems - Mammals: investigate how microplastics cause harm to large filter feeders <p><i>Overriding:</i></p> <ul style="list-style-type: none"> - Evaluate the types and size of litter ingested versus development stage |
|---|

Effects (lethal or sub lethal) under different environmental conditions of entanglement, in particular on threatened and protected species, (iii) Understanding how litter ingested by marine organisms, in particular threatened and protected species, affects their physiological condition and chemical burden, reduces survival and reproductive performance and ultimately affects their populations or communities, and (iv) Developing an Ecological Quality

Table 2
Recommendations for monitoring and research on litter ingestion by large marine organisms within MSFD.

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| <p>A) Monitoring</p> <p><i>Turtles (high priority):</i></p> <ul style="list-style-type: none"> - Define an EcoQO for the loggerhead turtle <i>Caretta caretta</i> in the Mediterranean sea (High priority) - Organize monitoring at sub regional (basin level) in a harmonised manner - Improve the collection of information/samples (stranded turtles and from fishermen) <p><i>Birds:</i></p> <ul style="list-style-type: none"> - Evaluate the potential value of nested litter as an indicator for the evaluation of GES and as a monitoring tool <p><i>Fish:</i></p> <ul style="list-style-type: none"> - Evaluate the potential value of fishes for monitoring the ingestion of litter by marine organisms <p>B) Research</p> <p><i>Birds:</i></p> <ul style="list-style-type: none"> - Identify bird species suitable for the development of a Fulmar type EcoQo - Better understand impacts of litter on nesting birds <p><i>Turtles:</i></p> <ul style="list-style-type: none"> - Better understand migrations of turtles in the Mediterranean. - Better understand how litter is affecting organisms (digestion, physiology, reproduction, population dynamics, etc.) <p><i>Mammals and sharks:</i></p> <ul style="list-style-type: none"> - Understand interactions between long term marine environmental changes and litter effects on mammals for the assessment of the quality of pelagic marine ecosystems - Mammals: investigate how microplastics cause harm to large filter feeders <p><i>Overriding:</i></p> <ul style="list-style-type: none"> - Evaluate types and size of litter ingested in relation to the stage of development |
|---|

Objective (EcoQO) for ingestion of litter in indicator species suitable for monitoring (sea turtles) (Tables 1 and 2).

5. Conclusions

In view of our current level of understanding of the harm to marine life caused by marine litter and the potential of large vertebrates for assessing Good Environmental Status, the above recommendations may help support the implementation of monitoring in the MSFD framework.

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Corresponding Author: Dr. Giuseppe A de Lucia, Ph.D.

Corresponding Author's Institution:

First Author: Giuseppe A de Lucia, Ph.D.

Order of Authors: Giuseppe A de Lucia, Ph.D.; Ilaria Caliani; Stefano Marra; Andrea Camedda; Stefania Coppa; Luigi Alcaro; Tommaso Campani; Matteo Giannetti; Daniele Coppola; Anna M Cicero; Cristina Panti; Matteo Baini; Cristiana Guerranti; Letizia Marsili; Giorgio Massaro; Maria C Fossi; Marco Matiddi

Abstract: A plethora of different sampling methodologies has been used to document the presence of micro-plastic fragments in sea water. European Marine Strategy suggests to improve standard techniques to make future data comparable. We use Manta Trawl sampling technique to quantify abundance and distribution of micro-plastic fragments in Sardinian Sea (Western Mediterranean), and their relation with phthalates and organochlorine in the neustonic habitat. Our results highlight a quite high average plastic abundance value (0.15 items/m³), comparable to the levels detected in other areas of the Mediterranean. "Site" is the only factor that significantly explains the differences observed in micro-plastic densities. Contaminant levels show high spatial and temporal variation. In every station, HCB is the contaminant with the lowest concentration while PCBs shows the highest levels. This work, in line with Marine Strategy directives, represents a preliminary study for the analysis of plastic impact on marine environment of Sardinia.

Research highlights

- ▶ Manta Trawl was used to estimate micro-plastic abundance on Sardinian Sea surface
- ▶ Micro-plastic densities and contaminants levels show high variability
- ▶ Hydrodynamic conditions can affect the level of plastic density

1 **Amount and distribution of neustonic micro-plastic off the Western Sardinian**
2 **coast (Central-Western Mediterranean Sea)**

3
4 Giuseppe Andrea de Lucia ^{a*}, Ilaria Caliani ^b, Stefano Marra ^a, Andrea Camedda ^{a,c}, Stefania Coppa
5 ^a, Luigi Alcaro ^e, Tommaso Campani ^b, Matteo Giannetti ^{b,d}, Daniele Coppola ^b, Anna Maria Cicero
6 ^e, Cristina Panti ^b, Matteo Bains ^b, Cristiana Guerranti ^b, Letizia Marsili ^b, Giorgio Massaro ^f, Maria
7 Cristina Fossi ^b and Marco Matiddi ^e

8
9 ^a *National Research Council – IAMC-CNR Oristano Section, Italy*

10 ^b *Department of Physical, Earth and Environmental Sciences, University of Siena, Via Mattioli, 4,*
11 *53100 Siena, Italy*

12 ^c *Tuscia University of Viterbo, Via S.M.in Gradi 4, 01100, Viterbo, Italy*

13 ^d *Department of Life Sciences, University of Siena, Siena, Italy*

14 ^e *ISPRA, National Institute for Environmental Protection and Research Rome, Italy*

15 ^f *Penisola del Sinis – Isola di Mal di Ventre MPA, Cabras, Italy*

16
17 * Corresponding author. Address: IAMC-CNR Institute for Coastal Marine Environment – National
18 Research Council, loc. Sa Mardini, 09170 Oristano, Italy.

19 Telephone: +39 0783 229015; Fax: +39 0783 229135

20 E-mail address: giuseppe.delucia@cnr.it

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26 **Abstract**

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28 A plethora of different sampling methodologies has been used to document the presence of micro-
29 plastic fragments in sea water. European Marine Strategy suggests to improve standard techniques
30 to make future data comparable. We use Manta Trawl sampling technique to quantify abundance
31 and distribution of micro-plastic fragments in Sardinian Sea (Western Mediterranean), and their
32 relation with phthalates and organochlorine in the neustonic habitat. Our results highlight a quite
33 high average plastic abundance value (0.15 items/m³), comparable to the levels detected in other
34 areas of the Mediterranean. “Site” is the only factor that significantly explains the differences

35 observed in micro-plastic densities. Contaminant levels show high spatial and temporal variation. In
36 every station, HCB is the contaminant with the lowest concentration while PCBs shows the highest
37 levels. This work, in line with Marine Strategy directives, represents a preliminary study for the
38 analysis of plastic impact on marine environment of Sardinia.

39

40 **Key words:** micro-plastics, marine litter, Marine Strategy, Sardinia, environmental impact,
41 pollution monitoring, phthalates

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43

44 **1. Introduction**

45

46 Plastics are synthetic organic compounds, derived from the polymerization of monomers extracted
47 from oil or gas (Rios et al., 2007). In the last fifty years, since these materials started to be utilized,
48 their presence in the marine environment has grown rapidly with the consequence that nowadays
49 40–80% of debris in the marine environment are plastic (Barnes et al., 2009; Cole et al., 2011).

50 The sources of plastic debris in marine environment may be either land (e.g. domestic objects) or
51 marine-based (e.g. nylon nets and other fishing industry residual) (Andrady, 2011; Derraik, 2002;
52 Goldberg, 1997; Gregory, 2009) and the highest levels of plastic pollution are usually found nearby
53 heavy urbanized areas (Derraik, 2002). The distribution and abundance of plastic debris are strongly
54 influenced by hydrodynamics and show high spatial variability in both the open ocean and shoreline
55 waters (Barnes et al., 2009; Browne et al., 2010).

56 Oceanic currents lead to high dispersion patterns (Law et al., 2010; Martinez et al., 2009;
57 Maximenko et al., 2012), which in turn permit plastic materials to reach remote areas, like islands
58 or polar regions, distant from sources of pollution (Barnes et al., 2009; Derraik, 2002; Gregory,
59 2009; Zarlf and Matthies, 2010).

60 In the marine environment plastic material requires several centuries, or even thousands of years, to
61 degrade (Arthur et al., 2009; Barnes et al., 2009; Derraik, 2002; Goldberg, 1997; Gorman, 1993;
62 Hansen, 1990; Moore, 2008; O’Brine and Thompson., 2010; UNESCO, 1994; Zarlf et al., 2011).
63 Debris items are usually subdivided into different size categories: mega-debris (> 10 cm); macro-
64 debris (2-10 cm); meso-debris (2cm-5 mm) and micro-debris (<5mm) (Barnes et al., 2009). Micro-
65 plastic particles are largely represented by plastic items, such as scrubbers and industrial pellets,
66 that serve as precursors for manufactured plastic products (primary sources). A great amount of
67 marine micro-particles is also constituted by fragments and fibres, derived from the breakdown of
68 larger plastic products (secondary sources) (Hidalgo-Ruz et al., 2012). Plastic micro-fragments are

69 accumulating on the sea surface, especially within the neustonic habitat, and several publications
70 report increasing concentrations in oceans and seas (Collignon et al., 2012, Doyle et al., 2011; Fossi
71 et al., 2012; Law et al., 2010; Moore et al., 2001; Ryan et al., 2009).

72 Plastic pollution was initially seen as a merely aesthetic problem (Galgani et al., 2013, Gregory,
73 2009), but many studies over the past decades show how several marine animals are negatively
74 affected by the presence of plastic (Boerger et al., 2010; Derraik, 2002; Galgani et al., 2013),
75 mainly by entanglement and ingestion (Gregory, 2009; Laist, 1997; Thompson et al., 2009; Van
76 Franeker et al., 2011).

77 The consequence of plastic ingestion could be the release and absorptions of plastic additives such
78 as phthalates, used to enhance plastic performance. Phthalates have been related to a wide range of
79 adverse health effects in several animals. Certain phthalates, such as DEHP (di-(2-
80 ethylhexyl)phthalate) and its metabolite MEHP (mono-(2-ethylhexyl) phthalate)), are reproductive
81 toxicants, mainly disturbing the reproductive system (Foster et al., 2000). Plastic material can also
82 adsorb toxic chemicals present at low concentrations in the water column, in particular persistent
83 organic pollutant (POPs) such as HCB, PCBs, DDTs (Rios et al, 2007; Teuten et al., 2009) which
84 can be released and absorbed by the organism after the ingestion. All these chemical pollutants,
85 acting as endocrine disruptors, can potentially affect organisms and populations viability (Caserta et
86 al., 2013; Fossi et al., 2007).

87 A wide variety of approaches, such as selective, bulk and volume-reduced techniques, have been
88 used to identify and quantify micro-plastics. The Manta Trawl, a modified neustonic net with
89 buoyant wings to keep the net aperture at the sea and air interface, is the most commonly used
90 equipment for sea surface micro-litter analysis based on a volume-reduced methodology (Hidalgo-
91 Ruz et al., 2012). The hood deflects wave crests into the submerged net, therefore capturing a
92 measurable volume of micro-debris at the sea surface.

93 The European Commission (EC) released the Marine Strategy Framework Directive
94 (MSFD/2008/56/EC) which indicates the major contaminant issues related to marine environments
95 and prioritizes the topics to be investigated in order to reach a Good Environmental Status (GES).
96 Considering the increasing abundance of plastic debris in the sea, the EC chose “Marine Litter” as
97 one of the 11 environmental descriptors on the basis of which the marine environmental status is to
98 be estimated. The Marine Strategy describes GES as the condition when “*Properties and quantities*
99 *of marine litter do not cause harm to the coastal and marine environment*” (Galgani et al., 2010).
100 Nevertheless, a standard methodology for the analysis of micro-plastic abundance, distribution and
101 potential effects on organisms has not been published yet. A standard methodology must be

102 developed and agreed upon before monitoring and mitigation activities can be initiated to support
103 the EU MSFD requirements.

104 The main goal of this work was to investigate, for the first time, the distribution of neustonic micro-
105 plastics in the area nearby the Gulf of Oristano (Sardinia), developing an integrated analytical
106 approach. Levels of phthalates and POPs were also estimated, in order to determine if there is a
107 correlation between these contaminants and micro-plastics density.

108

109 **2. Material and methods**

110

111 **2.1 Study area**

112 This study was carried out in the Gulf of Oristano and in an off-shore site nearby, in the western
113 sector of Sardinia (Central-Western Mediterranean Sea) (Fig.1). The Gulf of Oristano is a semi-
114 enclosed basin connected to the Sardinian Sea through a 9 km long opening delimited by Cape San
115 Marco on the north and Cape Frasca on the south. Two large lagoon systems are present: the Cabras
116 Lagoon on the northern part of the basin and the Marceddì lagoon on the southern part. Both lagoon
117 systems discharge their water into the gulf through their respective inlets. Another water input-point
118 from the surrounding mainland is the Tirso's river mouth located near the industrial harbour of
119 Oristano city. The typical wind patterns are the Mistral from north-west (NW), the Libeccio from
120 south-west (SW) and the Sirocco from south-east (SE). The Mistral can be considered the main
121 wind force acting in the area. The study area is included in the Algerian Basin, that presents
122 strongly different dynamics mainly constituted by cyclonic eddies (Olita et al., 2013).

123

124 **2.2 Sampling technique**

125 Thirty samples of neuston-plankton were collected using a Manta Trawl lined by a 500 µm mesh
126 net. Manta Trawl sampled the top 50 cm of the sea surface at an average speed of 2 knots for 20
127 minutes. The sampling activities were conducted only with Mistral blowing conditions, when wind
128 velocity was maximum 8 knots, in order to avoid the mixing of plastic particles in the water column
129 (Kukulka et al., 2012). The Manta Trawl was always towed against the wind. No data are available
130 for current's conditions at the time of sampling at a small spatial scale. The volume of filtered sea-
131 water (m³) was evaluated by a flow meter (MF315, *OceanTest Equipment, Inc.*). Samplings were
132 conducted in 4 coastal sites (Mal di Ventre – MDV, Caletta – CAL, Marceddì – MAR, Tirso – TIR,
133 Fig. 1) within 12 nautical miles (Nm) in consecutive days at the beginning of July 2012 and July
134 2013. Off-shore samples (i.e. 20 Nm out) were also collected on the 20th July 2013, during
135 “Minerva” sampling survey (MIN). The off-shore samples were collected in the morning (MIN D)

136 and during the night (MIN N) of the same day. For the estimation of the micro-plastic density, three
137 replicates were collected for every site and for every temporal sampling. In one of the three
138 replicates, the plastic component was removed and the remaining part was used for the estimation
139 of phthalates and organochlorine levels: for each site, samples were filtered by a 500 μm mesh net,
140 stored in liquid nitrogen and then analysed for contaminants.

141

142 **2.3 Micro-plastic evaluation**

143 For every sample, plastic items were separated from plankton and other organic matter, sorted and
144 measured under a binocular stereoscope (*AxioCam ERc5s for image analysis, Carl Zeiss*
145 *MicroImaging GmbH, Germany. www.zeiss.de/axiocam*), and only micro-materials (less than
146 5mm) were considered. Plastic items density was expressed as items/ m^3 .

147

148 **2.4 Phthalates analysis**

149 Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment. In both
150 invertebrates and vertebrates, DEHP is rapidly metabolized in the form of its primary metabolite,
151 MEHP (mono-(2-ethylhexyl) phthalate), which can be used as a marker of exposure to DEHP
152 (Barron et al., 1989). DEHP and MEHP were analysed in the neustonic/planktonic samples
153 following a method already described by Guerranti et al. (2013) and applied in plankton analysis by
154 Fossi et al. (2012), with improvement of QA/QC described by Guo and Kannan (2012) and
155 Schecter et al. (2013).

156 Each sample was thawed and weighed, and acetone was added. The resulting mixture was
157 sonicated, stirred and centrifuged. Then, the supernatant was placed in a further 15 ml tube and
158 precipitant was again added to 1 ml of acetone, sonicated, agitated and centrifuged for a further
159 separation. The supernatant phase was then recovered and rebuilt with what resulted from the first
160 extraction. The supernatants were then mixed and evaporated in a centrifugal evaporator. The
161 extract was re-suspended with 0.5 ml of acetonitrile and passed through a nylon filter with pores of
162 2 μm . Subsequently, the sample was placed in an auto-sampler vial and injected into an LC-ESI-MS
163 system. The instrumental analysis was performed with a Finnigan LTQ Thermo LC/MSn 110 with
164 an ESI interface. A total of 5 μl of the extracted sample was injected via the auto-sampler into the
165 HPLC system. A HPLC column Wakosil 3C18 was used. The mobile phases consisted of 100%
166 acetonitrile (A) and 0.05% aqueous acetic acid (B). Elution was performed using an isocratic mode
167 (A/B: 15/85, v/v) at 0.25 ml/min. ESI-MS was operated in the negative or positive ion mode
168 depending on the analytes (MEHP was detected in the negative mode, whereas DEHP was detected
169 in the positive mode). The heated capillary and voltage were maintained at 500°C and ± 4.0 kV,

170 respectively. For the quantitative analysis, a five-point calibration curve, prepared by the
171 progressive dilution of a solution of the two analytes of interest, was used. Following the
172 indications of Guo and Kannan (2012) and Schecter et al. (2013), three procedural blanks were
173 analysed with each set of five samples as a check for possible laboratory contamination and
174 interference. When the concentrations of DEHP in the three procedural blanks varied widely, and if
175 the difference in concentrations among the blanks exceeded 30 ng, then all the data were discarded,
176 and samples were reanalysed. Mean blank values were subtracted from sample values for each
177 batch. The data quality assurance and quality control protocols also included a daily calibration
178 verification. The limits of detection (LODs) for the compounds analysed were the values of the
179 compound in the blanks +3 SD. The LODs were 1.5 and 9 ng/g, respectively, for MEHP and
180 DEHP. Throughout this paper the levels of analytes below the limits of detection (<LOD) were
181 specified as values equal to the value of the LOD. Values were expressed in ng/g fresh weight
182 (f.w.).

183

184 **2.4 Organochlorines analysis**

185 Analysis for organochlorines (OC) compounds (HCB, DDTs and PCBs) were performed according
186 to the “U.S. Environmental Protection Agency (EPA) 8081/8082” method with modifications (Fossi
187 et al., 2002). Neustonic-planktonic samples were lyophilized in an Edwards freeze drier for 2 days
188 and about 0.25 g of sample were extracted with n-hexane for gas chromatography (Merck) in a
189 Soxhlet apparatus for analysis of organochlorines compounds. Whatman cellulose thimble (i.d. 25
190 mm, e.d. 27 mm, length 100 mm) used for extraction of the sample was preheated for about 30 min
191 to 110° C and pre-extracted for 9 h in a Soxhlet apparatus with n-hexane, in order to remove any
192 organochlorine contamination. The sample was spiked with surrogate compound (2,4,6-
193 trichlorobiphenyls - IUPAC number 30, Ballschmiter and Zell, 1980) prior to extraction. This
194 compound was quantified and its recovery calculated. The sample was extracted with n-hexane in
195 the thimble in the Soxhlet apparatus for 9 h. The sample was then purified with sulphuric acid
196 (Murphy, 1972) to obtain the first lipid sedimentation. The extract then underwent liquid
197 chromatography on a column containing Florisil that had been dried for 1 h in an oven at 110°C.
198 This further purified the apolar phase of lipids that could not be saponified, such as steroids like
199 cholesterol. Decachlorobiphenyl (DCBP - IUPAC number 209) was used as an internal standard,
200 added to each sample extracted prior to the analysis, and included in the calibration standard, a
201 mixture of specific compounds (Aroclor 1260, HCB and pp’- and op’-DDT, DDD and DDE). The
202 analytical method used was High Resolution Capillary Gas Chromatography with a Agilent 6890N
203 and a 63Ni ECD and an SBP-5 bonded phase capillary column (30 m long, 0.2 mm i.d.). The carrier

204 gas was N₂ with a head pressure of 15.5 psi (splitting ratio 50/1). The scavenger gas was
205 argon/methane (95/5) at 40 ml/min. Oven temperature was 100°C for the first 10 min, after which it
206 was increased to 280°C at 5C°/min. Injector and detector temperatures were 200°C and 280°C
207 respectively. The extracted organic material (EOM%) from freeze-dried samples was calculated in
208 all samples. Capillary gas-chromatography revealed op'- and pp'- isomers of DDT and its
209 derivatives DDD and DDE, and 30 PCB congeners. Total PCBs were quantified as the sum of all
210 congeners (IUPAC no. 95, 101, 99,151, 144, 135, 149, 118, 146, 153, 141, 138, 178, 187, 183, 128,
211 174, 177, 156, 171, 202, 172, 180, 199, 170, 196, 201, 195, 194, 206). These congeners constituted
212 80% of the total peak area of PCBs in the sample. Total DDTs were calculated as the sum of
213 op'DDT, pp'DDT, op'DDD, pp'DDD, op'DDE and pp'DDE. The results were expressed in ng/g dry
214 weight (d.w.). The detection limit was 0.1 ng/kg (ppt) for all the OCs analysed. The mean water
215 content was 85.9% (SD=4.5).

216

217 **2.5 Statistical analysis**

218 The analysis of variance on micro-plastic abundance among samples was conducted using the
219 statistical software PRIMER6 (*Plymouth Marine Laboratory*, UK) with PERMANOVA+ add-on
220 package (Anderson et al., 2008) on square root transformed data. Permutational ANOVA was
221 implemented using 9999 permutations, this routine was always associated with a Monte Carlo's
222 test, which constructs an asymptotic permutation distribution for the pseudo-F statistics allowing a
223 robust analysis even in the event that too few unique permutations are possible (Anderson et al.,
224 2008). Different experimental designs was implemented: "year" (two levels: 2012, 2013) was
225 treated as fixed factor while "site" (5 levels: MDV, CAL, MAR, TIR, MIN) was treated as random
226 and orthogonal to "year". This experimental design was also repeated excluding the off-shore
227 samples, i.e. using only 4 levels for the factor "site" (MDV, CAL, MAR, TIR). A second
228 experimental design, in which "time" (two levels: Day, Night) was treated as fixed factor, was used
229 to evaluate small-scale temporal differences on the distribution of micro-litter, using samples
230 collected on the off-shore station during day hours and few hours later, by night. Finally a third
231 experimental design was implemented in order to evaluate differences between shoreline and off-
232 shore samples: factor "distance" was treated as fixed, with two levels (shoreline, off-shore) while
233 factor "site" (with five levels: MDV, CAL, TIR, MAR, MIN), was treated as random and nested in
234 "distance". When PERMANOVA detected significant differences, post-hoc pairwise tests were run.
235 Data were tested for homogeneity of dispersion using Permutational Analysis of Multivariate
236 Dispersions (PERMDISP) based on Euclidean distance.

237

238 3. Results

239 All the samples analysed contained micro-plastic particles of different typology, described as sheet-
240 like, hard plastic, foam, filaments, polystyrene and row pellets. A total of 2673 micro-plastics were
241 collected in 18418.82 m³ of filtered sea-water, with an average concentration of 0.15 items/m³. In
242 the shoreline stations, mean concentration values assessed during the two sampling surveys were
243 0.10±0.04 items/m³ for MDV, 0.01±0.00 for CAL, 0.18±0.03 for MAR and 0.14±0.08 for TIR. The
244 highest numbers of items were found in the off-shore site, with average concentrations of 0.25±0.15
245 items/m³ for day-time samples (MIN D) and 0.35±0.11 items/m³ for night-time samples (MIN N).
246 PERMANOVA showed that spatial location of the different sampling sites can significantly
247 influence abundance of micro-particles (p=0.04). Even when off-shore station was excluded and the
248 analysis was conducted only among MDV, CAL, MAR and TIR sites, p-value associated with
249 factor “site” remained significant (Tab 1). For every sampling site, variations observed on micro-
250 plastic debris densities between 2012 and 2013 were not statistically significant. The differences
251 observed between off-shore and shoreline sites were not significant. The samples collected during
252 the day on “Minerva” sampling survey were similar to samples collected at night (p=0.48).

253 All the samples analysed for phthalates showed the presence of both DEHP and its metabolite
254 MEHP. The concentration of DEHP were very low, ranging from 9.00 ng/g f.w (LOD) to 13.74
255 ng/g f.w. In contrast, highest levels of MEHP were measured in the same samples, with the highest
256 value in MAR 2012 (30.99 ng/g f.w). Samples showed high variability differences both for the year
257 of sampling and for station location.

258 HCB, DDTs and PCBs levels were different depending on sampling sites. In every station, HCB
259 was the contaminant with the lowest concentration. Between 2012 and 2013 samples, MAR showed
260 remarkable differences in the levels of all OCs considered. In 2012 HCB and PCBs were not
261 detectable and DDTs were present at low levels (112.00 ng/g d.w.) while in 2013 all the three
262 compounds were detected, in particular the DDT concentrations were six times higher (665,3 ng/g
263 d.w) and the PCBs levels were about 2 ppm (1889.6 ng/g d.w.). Variable values were also present
264 in 2012 and 2013 samples from TIR station, with the highest values in 2012 (tab. 2). In MIN
265 station, the sample collected during the night presented levels of HCB, DDTs and PCBs markedly
266 higher than those collected during the day. OCs were not detected in CAL because of a very low
267 amount of starting material.

268

269 4. Discussion and conclusions

270

271 According to the MSFD, within the Descriptor 10 (Marine Litter), the EC member states are
272 required to develop and subsequently apply new methods to identify and quantify micro-plastics in
273 the marine environment. Additionally, the assessment of distribution and abundance of micro-
274 plastics in the Mediterranean Sea is mandatory, due to the lack of information on the whole basin.
275 In order to fill this gap, this work focused on the evaluation of amount and distribution of micro-
276 plastics in western Sardinia and the concentration of contaminants (phthalates and OCs) in the
277 neustonic/planktonic samples.

278 Plastic litter density correlates strongly with human population (Barnes et al., 2009). Even if the
279 study area is located near to a semi-enclosed basin where some input zones from the inland are
280 present, the low human density and the lack of strong industrial activities should support the
281 hypothesis of low micro-plastics presence in the area. However our results highlighted a quite high
282 average abundance value ($0.15 \text{ particles/m}^3$), comparable to the data obtained by Collignon et al.
283 (2012) from a widest sampling area in the NW of the Mediterranean. These preliminary results
284 suggested that micro-plastics are widespread in the Mediterranean and are reaching areas far from
285 pollution sources, like Sardinia. When we focused on a smaller spatial scale, we founded that
286 micro-plastics abundance showed high spatial variability: the analysis showed that location of the
287 sampling sites in the study area was the only factor that can significantly explain the differences
288 observed. We found the highest amounts in the off-shore site, where a density of $0.30 \text{ particles/m}^3$
289 was detected.

290 For the shoreline stations, that were sampled during summer 2012 and 2013, MAR site showed the
291 highest plastic contamination with mean values of 0.18 items/m^3 . Marceddì station is located in the
292 southern part of the Gulf of Oristano, its high abundance values are probably due to an inter-annual
293 variability lower than those observed for the other shoreline stations (Fig. 2). The lowest density of
294 plastics was present on CAL site ($0.01 \pm 0.00 \text{ items/m}^3$), which is located on Cape San Marco, the
295 promontory delimiting the Gulf on its northern part. Hydrodynamic features in this site are peculiar
296 and this may be the reason of such low (and low variable) values for this site, that pairwise test
297 showed as significantly different from the other sites (Tab. 3). Recent studies (see e.g. Olita et al.,
298 2013) highlight the presence of upwelling phenomena in the west coast of Sardinia, phenomena that
299 are, however, still poorly investigated. Upwelling may determine a dilution of plastic density on the
300 water surface and, therefore, stations in proximity of such a kind of phenomena (e.g. CAL site) may
301 present levels of plastic that are lower than the sites exposed to more stable conditions. Further
302 studies that clarify coastal upwelling phenomena and correlate them with plastic abundance are
303 suggested.

304 Pairwise test, conducted between different shoreline stations on the average between 2012 and 2013
305 values, shows that sites differ from each other, with the exception of TIR. TIR station presents high
306 variability among replicates that determine high standard error values. TIR station is located in the
307 proximity of the Tirso river's mouth and of the harbour of Oristano, hence it would be reasonable to
308 imagine that its position had a positive influence on the level of micro-plastic on the water surface.
309 Even if the correlation with micro-plastic density was not clearly detected, the presence of an
310 harbour and a river can determine peculiar hydrodynamics and therefore explain the high variability
311 detected for micro-plastic levels in TIR station.

312 The results showed that micro-plastics are ubiquitous in the sampling area. The amount of litter for
313 the off-shore station did not differ from the litter amount collected during 2013 sampling campaign
314 on shoreline stations (MDV, CAL, MAR, TIR). This could be due to the high dispersion of values,
315 both from off-shore than from shoreline stations, around the sampling average (standard error). The
316 results highlighted an high variability also for the concentrations of contaminants, both among sites
317 and among samples from the two different sampling campaigns. Phthalates were markedly different
318 among the samples collected in 2012 and 2013. In particular, higher levels of MEHP and
319 undetectable levels of DEHP in the first sampling were found. On the other hand, MEHP was
320 lower, and DEHP was detectable in the second one. This could be reasonably attributed to a
321 different composition of the plastics present in the environment (a feature that will definitely be
322 assessed in the future through qualitative analysis of plastics present in the sampling site), to a
323 change in the degree of contamination of the aqueous medium or even to a more recent exposure of
324 organisms with the DEHP, which could be considered less metabolized than in the previous
325 scenario. However, especially for phthalates, such variability needs to be considered normal, given
326 the extreme rapidity with which the DEHP, and its metabolite MEHP are eliminated by organisms.
327 To the authors' knowledge, there are no data in the literature showing that plankton has the capacity
328 to metabolize DEHP. However, in addition to vertebrates, some studies highlighted that phthalates
329 could be degraded also by microorganisms and invertebrates (Albro et al., 1993; Taylor et al.,
330 1981). In humans, for example, DEHP is rapidly cleared from the body with a short half-lives in the
331 order of a few dozen hours (Preau et al., 2010; Völkel et al., 2002). Regarding the levels of DEHP
332 and MEHP, these, on average, tend to be lower than those previously found by Fossi et al. (2012) in
333 plankton samples collected in Sardinia and Liguria (Italy); the same study confirms the high
334 variability of the levels found among samples collected in the same area. Additionally, to better
335 understand if the level of chemicals in plankton are related to the ingestion of particles more than to
336 dissolved chemicals other aspects need to be considered such as: phthalates sources (e.g.

337 atmospheric and degradation processes), the mechanisms of dispersion and leaching of phthalates
338 and the chemicals reactions occurring in the environment.

339 The results obtained in the present study demonstrate the need to perform a high number of
340 replicates for each sampling site due to the variability of this kind of environmental issue.

341 In conclusion, in order to respond to MSFD requirements, this work underlines the importance to
342 use an integrated and consistent approach combining data on distribution and abundance of micro-
343 plastics with contaminants adsorbed to, or released by plastic. This will allow more reliable
344 measures to understand the trend of marine litter contamination and to eventually recommend
345 mitigation actions to reach the Good Environmental Status of the Mediterranean Sea.

346

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539 Table 1. Results of PERMANOVA on square root transformed data. df: degree of freedom; SS:
 540 Sum of Squares; MS: Mean Square; Pseudo-F: value of the Pseudo-F statistic; P: p-value; n.s.: non-
 541 significant. Significant results are reported in bold.

| PERMANOVA | | | | | | | | |
|-------------|---------|--------|--------|----|------|------|----------|-----------------|
| Name | Abbrev. | Type | Levels | df | SS | MS | Pseudo-F | P |
| YEAR | YE | Fixed | 2 | 1 | 0.06 | 0.06 | 1.90 | n.s. |
| SITE | SI | Random | 5 | 4 | 0.40 | 0.10 | 3.20 | <0.05 |
| YEAR X SITE | YExSI | | | 3 | 0.08 | 0.03 | 0.94 | n.s. |
| PERMDISP | | | | | | | | n.s. |
| YEAR | YE | Fixed | 2 | 1 | 0.06 | 0.06 | 1.90 | n.s. |
| SITE | SI | Random | 4 | 3 | 0.30 | 0.10 | 5.27 | <0.01 |
| YEAR X SITE | YExSI | | | 3 | 0.08 | 0.03 | 1.55 | n.s. |
| PERMDISP | | | | | | | | n.s. |
| TIME | TI | Fixed | 2 | 1 | 0.05 | 0.05 | 0.61 | n.s. |
| PERMDISP | | | | | | | | n.s. |
| DISTANCE | DI | Fixed | 2 | 1 | 0.10 | 0.10 | 1.22 | n.s. |
| SITE (DI) | SI | Random | 5 | 3 | 0.19 | 0.06 | 1.44 | n.s. |
| PERMDISP | | | | | | | | n.s. |

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 543

544 Table 2. Mean micro-plastic items density and levels of contaminants (phthalates and
 545 organochlorines) for each sampling station (MDV: Mal di Ventre; CAL: Caletta; MAR: Marceddì;
 546 TIR: Tirso; MIN D: Minerva/off-shore day-time MIN.N: Minerva/off-shore night-time). Values are
 547 expressed as items/m³ for micro-plastics; as ng/g dry weight for phthalates and as ng/g fresh weight
 548 for organochlorines. Starting-ending coordinates and volume of water filtered (express as m³) are
 549 reported for every replicate.
 550

| sampling station | replicate | starting point | ending point | m ³ | items/m ³ | ng/g | | | | | |
|------------------|-----------|------------------------|------------------------|----------------|----------------------|-------|---------|---------|-------|-------|--|
| | | | | water filtered | micro-plastic | HCB | DDTs | PCBs | MEHP | DEHP | |
| MAR 2012 | a | N39 46 23.7 E8 29 59.6 | N39 47 12.4 E8 29 30.5 | 827 | 0.20 ± 0.03 | n.d. | 112,00 | n.d. | 30,99 | 9,00 | |
| | b | N39 46 21.5 E8 29 35.6 | N39 47 05.7 E8 29 11.2 | 729 | | | | | | | |
| | c | N39 46 24.7 E8 29 31.6 | N39 47 09.1 E8 29 05.3 | 729 | | | | | | | |
| MAR 2013 | a | N39 45 36.5 E8 28 59.9 | N39 46 11.4 E8 29 17.7 | 438 | 0.17 ± 0.06 | 8,10 | 665,30 | 1889,60 | 2,56 | 10,24 | |
| | b | N39 45 28.9 E8 28 51.4 | N39 46 3.2 E8 29 9.6 | 347 | | | | | | | |
| | c | N39 45 35.8 E8 28 58.0 | N39 46 5.5 E8 29 24.06 | 357 | | | | | | | |
| TIR 2012 | a | N39 52 41.0 E8 32 04.2 | N39 52 18.0 E8 30 45.2 | 924 | 0.09 ± 0.08 | 8,90 | 2130,10 | 3793,10 | 25,62 | 9,00 | |
| | b | N39 52 23.4 E8 31 26.0 | N39 51 58.9 E8 30 22.0 | 754 | | | | | | | |
| | c | N39 52 21.2 E8 31 26.7 | N39 51 57.7 E8 30 26.8 | 707 | | | | | | | |
| TIR 2013 | a | N39 53 9.1 E8 32 18.2 | N39 53 17.7 E8 31 29.1 | 454 | 0.19 ± 0.15 | 4,90 | 547,80 | 2256,60 | 1,64 | 9,63 | |
| | b | N39 53 5.3 E8 32 6.9 | N39 53 22.9 E8 31 38.0 | 323 | | | | | | | |
| | c | N39 53 7.9 E8 32 17.0 | N39 53 30.5 E8 31 51.0 | 401 | | | | | | | |
| MDV 2012 | a | N40 00 00.6 E8 18 30.3 | N40 00 25.2 E8 17 39.8 | 868 | 0,021 ± 0,00 | - | - | - | - | - | |
| | b | N40 00 21.3 E8 17 38.7 | N40 00 34.9 E8 16 39.8 | 868 | | | | | | | |
| | c | N40 00 13.8 E8 16 43.9 | N40 00 22.4 E8 15 44.7 | 814 | | | | | | | |
| MDV 2013 | a | N39 59 51.4 E8 17 15.6 | N39 59 19.7 E8 17 19.7 | 631 | 0.19 ± 0.04 | 13,70 | 1166,60 | 3224,50 | 3,15 | 13,74 | |
| | b | N40 0 2.1 E8 17 26.9 | N39 59 25.0 E8 17 16.6 | 537 | | | | | | | |
| | c | N39 59 50.1 E8 17 12.7 | N39 59 10.1 E8 17 14.6 | 596 | | | | | | | |
| CAL 2012 | a | N39 52 34.4 E8 27 18.0 | N39 51 45.9 E8 26 42.5 | 850 | 0.01 ± 0.00 | - | - | - | 16,45 | 9,00 | |
| | b | N39 51 58.4 E8 27 24.2 | N39 51 19.4 E8 26 34.8 | 870 | | | | | | | |
| | c | N39 52 42.4 E8 27 06.0 | N39 51 44.6 E8 26 52.7 | 790 | | | | | | | |
| CAL 2013 | a | N39 51 49.9 E8 26 42.2 | N39 51 11.6 E8 26 33.6 | 467 | 0.02 ± 0.01 | - | - | - | 3,45 | 11,14 | |
| | b | N39 51 48.9 E8 26 38.0 | N39 51 16.6 E8 26 17.9 | 335 | | | | | | | |
| | c | N39 51 51.0 E8 26 33.4 | N39 51 24.0 E8 26 14.9 | 292 | | | | | | | |
| MIN D | a | N39 43 58.9 E8 03 07.2 | N39 44 39.1 E8 03 07.0 | 671 | 0.25 ± 0.15 | 0,64 | 185,40 | n.d. | 3,34 | 11,37 | |
| | b | N39 46 12.2 E8 03 07.5 | N39 46 52.3 E8 03 07.3 | 685 | | | | | | | |
| | c | N39 48 01.5 E8 03 07.2 | N39 48 40.2 E8 03 07.8 | 722 | | | | | | | |
| MIN N | a | N39 50 18.7 E8 02 43.7 | N39 49 34.2 E8 02 40.6 | 468 | 0.35 ± 0.11 | 8,30 | 760,30 | 2025,80 | 2,55 | 10,64 | |
| | b | N39 48 14.3 E8 02 31.3 | N39 47 30.8 E8 02 32.5 | 542 | | | | | | | |
| | c | N39 46 02.1 E8 02 37.2 | N39 45 23.0 E8 02 34.9 | 420 | | | | | | | |

553 Table 3. Pair-wise comparisons between sites (MDV: Mal di Ventre; CAL: Caletta; MAR:
 554 Marceddi; MIN: Minerva/off-shore) conducted on the average between 2012 and 2013 values of
 555 micro-plastic density. t: value of the test; P: p-value. Significant results are reported in bold.
 556

PAIR-WISE COMPARISON

| Groups | t | P |
|---------------|----------|------------------|
| MDV, CAL | 5,82 | <0.001 |
| MDV, MAR | 3,01 | <0.05 |
| CAL, MAR | 7,15 | <0.001 |
| CAL, MIN | 2,6 | <0.05 |

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560 **Figure captions:**

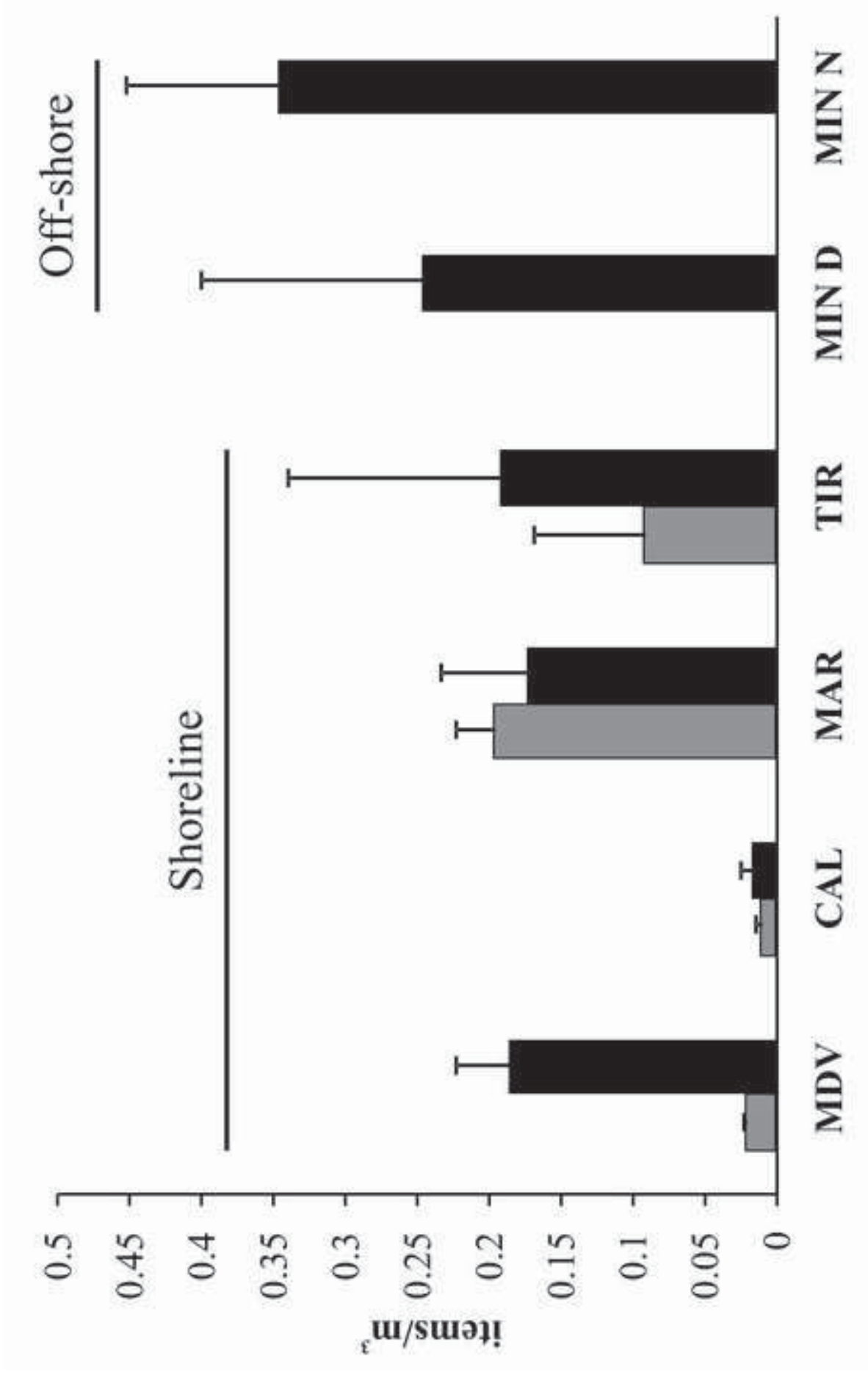
561 Figure 1. Study area with the different sampling stations. For each sampling station, year and time,
562 the levels phthalates (ng/g d.w.) and organochlorines (ng/g f.w.) are reported in relation to micro-
563 plastic density (items/m³). Different columns represent the percentage respect the maximum value
564 observed in the study area.

565 Figure 2. Micro-plastic density levels (mean \pm SE) for each sampling station (MDV: Mal di Ventre;
566 CAL: Caletta; MAR: Marceddi; TIR: Tirso; MIN D: Minerva/off-shore day-time MIN N:
567 Minerva/off-shore night-time). Grey columns: samples from 2012 campaign; Black columns:
568 samples from 2013 campaign.

Figure
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Short communication

Plastic litter in the sea

M.H. Depledge^a, F. Galgani^b, C. Panti^c, I. Caliani^c, S. Casini^c, M.C. Fossi^{c,*}^a European Centre for Environment and Human Health, University of Exeter Medical School, Heavitree Road, Exeter EX1 2LU, United Kingdom^b Ifremer, Immeuble Agostini, ZI Furiani, 20600 Bastia, Corsica, France^c Dipartimento di Scienze Fisiche, della Terra e dell'Ambiente, Università di Siena, Via P.A. Mattioli 4, 53100 Siena, Italy

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ABSTRACT

On June 2013 a workshop at the University of Siena (Italy) was organized to review current knowledge and to clarify what is known, and what remains to be investigated, concerning plastic litter in the sea. The content of the workshop was designed to contribute further to the European Marine Strategy Framework Directive (MSFD) following an inaugural workshop in 2012. Here we report a number of statements relevant to policymakers and scientists that was overwhelming agreement from the participants. Many might view this as already providing sufficient grounds for policy action. At the very least, this early warning of the problems that lie ahead should be taken seriously, and serve as a stimulus for further research.

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1. Introduction

Plastic litter is now almost ubiquitous in the World's oceans, extending from the coast far out to sea, and down onto the sea floor. Macroscopic plastic (bottles, plastic bags, old toys, etc.) is in evidence on most tourist beaches, in harbours and marinas, and can be readily spotted from the decks of ferryboats, cruise ships and leisure craft. One of the main causes of this global problem is increasing plastic production. The annual production has increased dramatically from 1.5 million tonnes in the 1950s to approximately 280 million tonnes in 2011. Microplastic fragments (smaller than 5 mm) potentially less obvious nanoscale plastic, is readily detectable in sand, sediment and even in marine biota. The latter may originate directly in the micro or nano forms, or result from the breakdown or abrasion of larger pieces of plastic. Microplastics have been accumulating in oceans globally over at least the last four decades and have invaded even the most remote marine environments. Knowledge about the effects of this micro-debris is limited, but nonetheless, a horizon scan of global conservation issues recently identified microplastics as one of the main global emerging environmental threats.

Numerous non-governmental organisations, wildlife charities and environmental agencies have drawn attention to the plastic litter issue, yet the scale of the problem is not widely appreciated by

the public or politicians. Few, if any, practical measures have been put in place to manage the situation. Concerns extend from the unsightliness of macroplastics affecting coastal tourism, to various effects on ecosystem structure and functions, through adverse impacts on particular species and even the death of individuals (Browne et al., 2011).

Recently, a significant positive relationship between microplastics abundance and human population-density was demonstrated. Since the human population continues to increase, the prevalence of microplastics will also probably increase (Rochman et al., 2013).

Academics and other researchers have now published several authoritative reports on the effects of plastic litter on marine birds, turtles, marine mammals and other marine vertebrates and invertebrates (Wright et al., 2013). Their studies have identified plastic fragments in the water column, in sandy and muddy sediment and in the guts, respiratory structures and tissues of marine species.

On the 5th and 6th June, 2013, ca. 100 scientists from 6 countries met for a workshop at the University of Siena, Italy, to review current knowledge and to clarify what is known, and what remains to be investigated, concerning plastic litter in the sea. The content of the workshop was designed to contribute further to the European Marine Strategy Framework Directive (MSFD) following an inaugural workshop in 2012 (see Fossi et al., 2012). In addition, a number of statements relevant to policymakers were prepared by the organisers at the end of the meeting which are presented here, and which had the overwhelming support of the workshop participants.

* Corresponding author. Tel.: +39 0577 232913; fax: +39 0577 232930.
E-mail addresses: panti4@unisi.it (C. Panti), fossi@unisi.it (M.C. Fossi).

2. What we know concerning plastic litter in the sea

Several key facts were highlighted at the workshop:

- 1) Plastic litter is diverse and now very widely distributed in the marine environment.
- 2) Many kinds of plastic litter are extremely persistent, often for several decades.
- 3) Some kinds of marine organisms are particularly vulnerable to plastic litter, including turtles, marine mammals, suspension feeders and deposit feeders.
- 4) Some plastics components and their constituent chemicals can be transferred through marine food webs (eg. phthalates).
- 5) Plastic litter can play a role in facilitating the introduction of invasive species into new localities, thereby influencing both biodiversity and ecosystem structure and functions in some areas.
- 6) The use of plastics is continuing unabated and will increase in the future.
- 7) Hydrodynamics and degradability determine the fate of litter at sea.
- 8) Policymakers, politicians and the public remain largely unaware of the extent of the problem and the magnitude of the threat to marine ecosystems.

3. Emerging questions

Following the presentation of research papers at the workshop and in subsequent discussions of each session, a number of questions were identified where further research is required to provide answers. They included the following:

- 1) How much plastic is getting into the marine environment each year?
- 2) What are the key sources?
- 3) What are geographic distributions of plastic litter of different sizes?
- 4) What are the relative proportions of macro, micro and nanoplastic entering the marine environment and which pose the greatest threat?
- 5) Where do the different types of plastic litter accumulate?
- 6) How long does each type persist?
- 7) Is plastic taken up by marine organisms?
- 8) Is it damaging to them? Is harm well understood?
- 9) Which kinds of marine organisms most impacted by macro and microplastics?
- 10) What are the mechanisms by which damage occurs?
- 11) How does plastic interact with other environmental pollutants and influence their toxicity?
- 12) What is the extent of economic, environmental and human health costs resulting from the presence of plastic litter in the marine environment?

4. What can be done?

During the course of the workshop, various needs and measures were discussed that might form the basis for beginning to address threats posed by plastic litter. They included:

- 1) the need to increase awareness of the scale and severity of the issue through public education programmes,
- 2) clear identification of who is responsible for managing plastic production and levels of release into the environment,

- 3) provision of guidelines on the safe disposal of plastics,
- 4) development of regulations to ensure the safe disposal of plastic – and their enforcement,
- 5) reduction of the use of plastics worldwide through international agreements,
- 6) finding environmentally friendly alternative to plastics,
- 7) development and implementation of programmes for the collection and proper disposal of plastics (for example, beach clean ups, collection for recycling and reuse, etc.),
- 8) monitoring trends and effects of marine litter at sea,
- 9) evaluation of the presence and effects of marine debris (particularly microplastic) in marine environment using marine organisms as sentinel species and applying new integrated monitoring tools.

5. Summary and conclusions

There is clearly much to be done to bring the issue of plastic litter in the seas to the attention of the public, policymakers and politicians. Fortunately, the European Commission and other funding organisations around the World have at last begun to support research work in this area (see for example, EU projects such as CLEANSEA, MICRO, PERSEUS, MARELITT, MARLISCO, KIMO, etc.). Nonetheless, positive action to curtail and manage of the use of plastics and their disposal is still urgently needed. In this regard, within the European MSFD a proper descriptor (Descriptor 10) was dedicated to marine litter. Task group 10 defines marine litter as “any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment” (Galgani et al., 2010) with a view to using mitigation measures to achieve the Good Environmental Status in European waters by 2020.

A recent initiative (July 2013) was proposed by the University of Siena, under the umbrella of the United Nations Sustainable Development Solutions Network MED Solution (directed by Professor Jeffrey D. Sachs – Earth Institute, Columbia University). The main objective of the Solution Project (PLASTIC-BUSTERS) will be to evaluate the presence and effects of marine debris (particularly microplastics) in the Mediterranean environment using marine organisms as sentinel species and applying a new integrated monitoring tool. The international project will help to reinforce existing Mediterranean international efforts to harmonize monitoring and mitigation activities in the entire basin.

In the final session of the Siena workshop, three statements were presented to the participants:

- i) Do you agree that there is robust scientific evidence that individuals of some species of marine organisms have already been adversely affected by plastic litter in the seas?
- ii) Do you agree that marine ecosystem services are being adversely affected by plastic litter?
- iii) Do you agree that there is robust evidence that plastic litter has, in some cases, damaged human health, wellbeing or prosperity?

There was overwhelming agreement from the participants (and no voices of dissent) as to the veracity of these statements. Many might view this as already providing sufficient grounds for policy action. At the very least, this early warning of the problems that lie ahead should be taken seriously, and as a stimulus for further research.

Further details of research into the impact of plastic litter in the marine environment and particularly the potential use of large

marine vertebrates (ranging from large pelagic fish, sea turtles, sea birds and cetaceans) in determining the environmental status of marine ecosystems (descriptors 1, 8 and 10 – Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008) will be reported in the proceedings of the 2013 workshop to be published in a special issue of *Marine Environmental Research* in 2014.

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