QUANTIFYING A MARINE ECOSYSTEM'S RESPONSE TO A CATASTROPHIC OIL SPILL

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SUMMARY: Crude oil production currently exceeds 4 billion tonnes per annum, with >1.3 million tonnes entering the marine environment each year. On the 10th September 2017 the vessel Agia Zoni II sank in the Saronic Gulf, Greece, releasing an estimated 2,500 tonnes of crude oil, which heavily contaminated the coasts of Salamina and the Athens Riviera. Sediment and water samples from contaminated beaches (and uncontaminated control sites) were taken shortly after the incident and thereafter monthly over the following seven months. Here, we will use a combination of gas chromatography mass spectrometry (GC-MS) and molecular techniques to measure changes in hydrocarbon composition and concentration in relation to the in situ microbial community composition. The overall aim of this project is to quantify the efficacy of the clean-up operation and the extent of the indigenous microorganisms for natural biodegradation of petroleum hydrocarbons. Specifically, we will monitor the effects of the oil spill on Bacterial, Archaeal, and Fungal communities, using qPCR and Next Generation Sequencing targeting phylogenetic and functional genes. This analysis will identify those microbes involved in hydrocarbon biodegradation of the pollutants, as well as those key functional groups of microbes that may have been impacted negatively (e.g. from direct toxicity or by being out-competed by oil-degrading microbes). Furthermore, this study aims to quantify any long-term effects of the oil spill on the wider marine food web, by measuring any differences in primary and secondary production. This incident has provided the rare opportunity to study a large oil spill *in situ*; enabling the direct measurement of the environmental effects, and such data may assist in the design of better oil remediation and management strategies in the future.

1. INTRODUCTION

Crude oil production currently exceeds 4 billion tonnes per annum, with >1.3 million tonnes entering the marine environment each year. Oil demand forecasts continue to grow, with the U.S Energy Information Administration predicting global inventories to increase by 0.6 million barrels of oil a day in 2019 (EIA, 2018). Oil pollution remains a significant threat to marine ecosystems, tourism, and fisheries. Europe is particularly vulnerable; with over half of the 20 biggest oil-shipping disasters occurring in Europe.

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On the 10th September, the Agia Zoni oil tanker sank in the Saronic Gulf (near Athens, Greece) releasing over 2,500 tonnes of fuel oil and marine gas into the marine environment (Fig. 1). The slick drifted 30 km along the Athens Riviera to Glyfada and Piraeus Port, contaminating the *in-situ* biota. Of concern are toxic PAHs which may sorbed to, or become buried in, sediments, and which are environmentally persistent and can bioaccumulate in the food chain. Specifically, 4- & 5-ring PAHs are highly recalcitrant and toxic to marine life (De La Huz *et al.*, 2005). Moreover, hydrocarbons locked in sediments are also likely to be released by bioturbation or dredging, with detrimental impacts on local (shell) fisheries.

Processes such as evaporation, dispersion, emulsification, and biodegradation, amongst others, will naturally remove a substantial proportion of hydrocarbons from the environment. However, the natural attenuation of crude oil in marine environments is affected by several factors, such as the oil type, weather and environmental conditions, as well as by the properties of the marine environment (Brandvik *et al.*, 2010). This oil-spill not only directly impacts species and habitats, but may also set off a cascade of perturbations affecting the entire food web (Short, 2017). The ramifications of oil spills may therefore be enormous, with the potential to fundamentally alter future ecosystem structure and functioning.

Bioremediation efforts are therefore often sought after, especially when, as is the case with Agia Zoni II, the oil-spill occurs along coastlines. Hydrocarbonclastic microorganisms , play a significant role in the biodegradation of petroleum hydrocarbons; (Atlas, 1981; Bossert & Bartha, 1984). Consequently, oil spills can dramatically alter marine microbial community composition, resulting in a decrease in species richness and diversity (Head *et al.*, 2006; McGenity *et al.*, 2012). Many microbes decrease in abundance, due to direct toxic effects from oil, or by being outcompeted for available resources (e.g. N, P). However, there is niche-partitioning between species in the utilisation of different hydrocarbon components (McKew *et al.*, 2007), with a strong selection for alkane-degrading genera e.g. *Alcanivorax*, *Thalassolituus*, *Oleibacter*, and PAH-degrading bacteria e.g. *Cycloclasticus* (Head *et al.*, 2006; McGenity *et al.*, 2012). Yet, we still know little about how community structure changes over time (as oil components are sequentially degraded, and nutrient status is altered), or which species dominate under particular environmental conditions. Since opportunities to study large oil spills *in situ* are rare, it is important for us to understand the effects of an oil spill in natural settings to enable the design of better oil remediation and management strategies for future spills and limit the damage they cause to coastal environments and marine life.

Figure 1. Impact of the Agia Zoni II tanker oil-spill located on the Salamina coastline, Greece. Image taken by Archipelagos Marine Conservation Institute, Greece.

2. AIM AND OBJECTIVES

The overall aim of this study is to better understand the impact of the Agia Zoni II tanker oil-spill in the Saronic Gulf in relation to the efficacy of the Greek oil-spill management response.

Our specific objectives are as follows:

1. Quantify composition and concentration of oil hydrocarbons over time.
2. Determine temporal changes in abundance, diversity, and composition of the *in-situ* microbial community (Bacteria/Archaea/Fungi/microeukaryotes), particularly hydrocarbon-degrading (such as *Alcanivorax*, *Thalassolituus*, *Oleibacter*, and *Cycloclasticus)*, in relation to hydrocarbon composition and concentration, nutrient availability and other environmental factors.
3. Determine potential wider-effects on the marine food web via measurements of primary and secondary productivity.

3. METHODOLOGY

3.1. Sampling Location

Sample locations were chosen at specific areas along the Athens Riviera and Salamina coast line to reflect both unimpacted (control) and oil-contaminated sites. In total, nine locations were sampled. Four contaminated sites along the Athens Riviera and three contaminated sites along the Salamina coast (Fig. 2). In addition, control (unimpacted) sites were also sampled at both the Athens Riviera and Salamina coast (Fig. 2). Triplicate 20g sediment and 100 ml water were taken (from the shore line), at all sites. Samples were subsequently frozen at -20°C, prior to shipment to the University of Essex, UK. Samples were collected within 7 days of the initial oil-spill (September 2017), and subsequently in the months of October, December, January, February, March, and April.

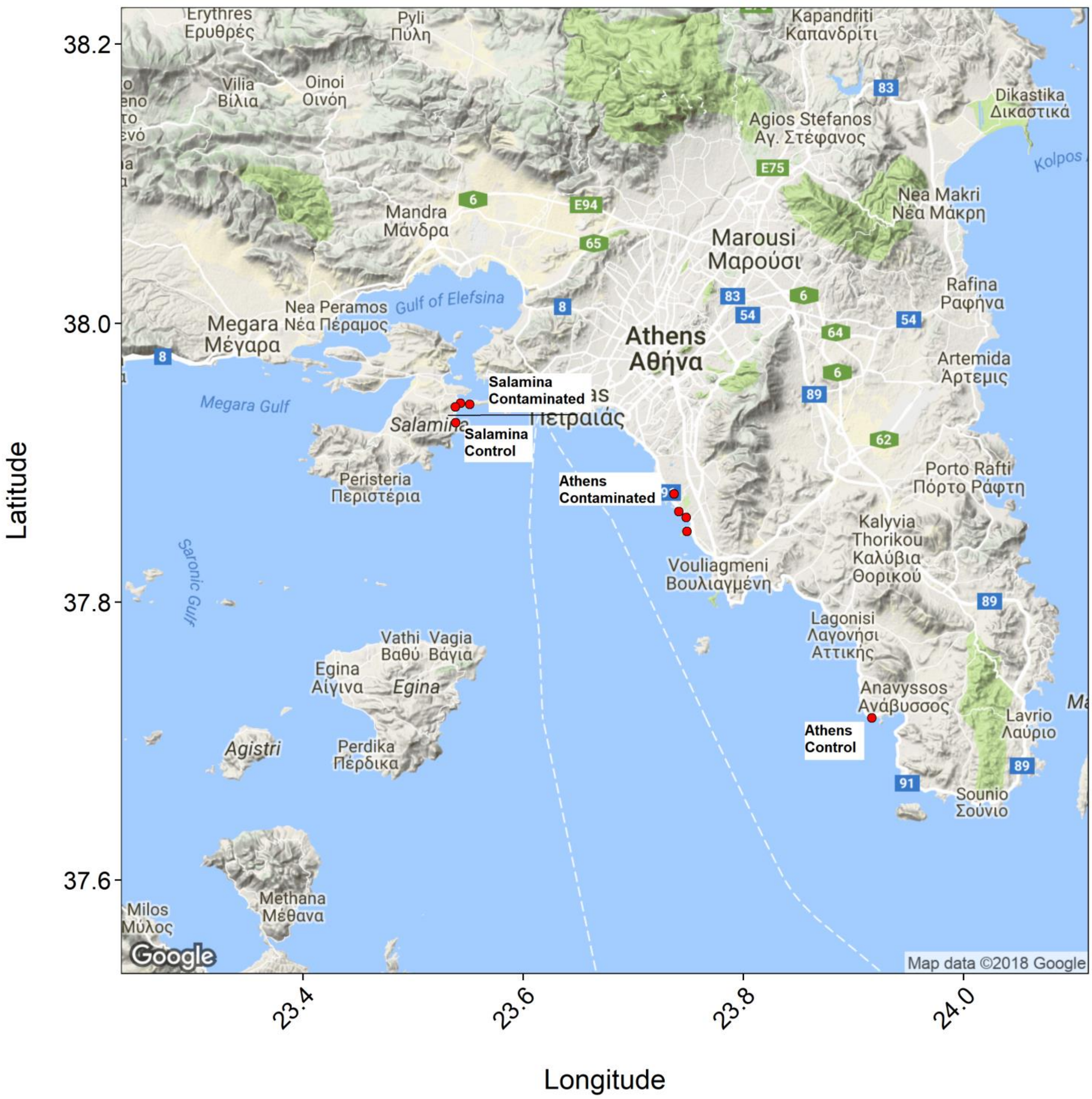

Figure 2. Map of the Athens Riviera and Salamina coast line (Greece) with sample sites marked.

3.2. Oil-degrading Microbial Community Succession

Over the coming months, we will analyse the collected samples to quantify the succession of oil-degrading taxa and changes in microbial community composition. DNA will be extracted from the sediment and water samples, using a DNeasy PowerSoil and PowerWater Kits (Qiagen) according to the manufacturer's instructions and qPCR will be conducted on a CFX384™ Real-Time PCR Detection System (BioRad) to measure absolute abundances of Bacteria, Archaea, and Fungi [using primers for bacterial 16S rRNA (Klindworth *et al.*, 2013), Fungal ITS (Ihrmark *et al.*, 2012) and archaeal 16S rRNA (Stahl D. A. & Amann, 1991; Raskin *et al.*, 1994)] as previously described (McKew & Smith, 2017).

3.2.1 Next Generation Sequencing

Metagenetic amplicon libraries will be prepared with the same primers as the qPCR primers, but flanked with Illumina Nextera overhang sequences as previously described (Landsdown *et al*., 2016) and sequenced on an Illumina MiSeq® platform, using MiSeq® v3 reagent kits.

3.2.2 Bioinformatics and Statistical Analysis

Sequence output from the Illumina MiSeq platform will be analysed within BioLinux. Firstly, sequence reads will be quality trimmed using Sickle (Joshi & Fass, 2011) prior to error correction within SPades (Nurk *et al.*, 2013) using the BayesHammer algorithm (Nikolenko *et al.*, 2013). Sequence reads will be pair-end aligned with PEAR (Zhang *et al.*, 2014) within PANDASeq (Masella *et al.*, 2012). The sequence reads will be dereplicated, sorted by abundance, and clustered into OTUs (Operational Taxonomic Units) at the 97% level using VSEARCH (Rognes *et al.*, 2016). Singleton OTUs will be discarded, as well as any chimeric sequences, using reference-based chimera checking using UCHIME within VSEARCH. Taxonomic assignment will be conducted with RDP Classifier (Wang *et al.*, 2007).

3.3. Hydrocarbon Analysis

Hydrocarbons will be solvent extracted from sediments and seawater with of hexane and dichloromethane (1:1). Deuterated alkanes and PAH internal standards will be added to the extracts. Aliphatic hydrocarbons and PAH will be identified and quantified by gas chromatography-mass spectrometry (GC-MS) as previously described (Coulon *et al.*, 2007).

3.4. Marine Food Web

In situ ecosystem productivity in impacted and control beaches was assessed by placing replicate clear acrylic domes in the subtidal nearshore habitats within 20 m of the shore and in approximately 1m of water depth. Each dome was customised to insert into the sediment to create a full seal over the sediment and fitted with a miniDOT O^2 and temperature data recorder (PME miniDOT) to assess oxygen evolution over a 6-hour period. By placing foil over the domes to induce a dark period net ecosystem productivity can be determined. This was undertaken in March and May 2018.

Ex situ ecosystem productivity effects of oil spills are assessed by incubating sediments from clean beaches in temperature controlled closed circuit vessels with and without inoculation of an oil sample from an impacted area. Productivity is measured as oxygen evolution (as above) but using optical probes (Vernier Optical DO Probe ODO-BTA). In addition, measurements of methane production were taken before and after the oil addition treatment versus the controls. Samples of the sediment were kept from before and after the control and oil spill addition experiments to link to the community succession analysis above.

4. OIL SPILL RESPONSE MANAGEMENT

The sinking of the Agia Zoni II, as well as other recent maritime accidents that have occurred in Greek waters, clearly demonstrates the lack of an efficient mechanism to prevent and respond to maritime accidents. The economic benefits which result from the growing shipping sector should not occur at the expense of the marine wealth and biodiversity, or of socio-economically vital sectors, such as tourism and fisheries that heavily depend on the clean waters. If pollution levels increase, or a shipping disaster occurs, the results could be devastating and long lasting, surpassing any economic gains from shipping transport activities in the region. Therefore, the introduction of efficient measures for the prevention and management of potential shipping accidents is of grave importance, as catastrophic events could have long-term, irreversible impacts on human and marine health.

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