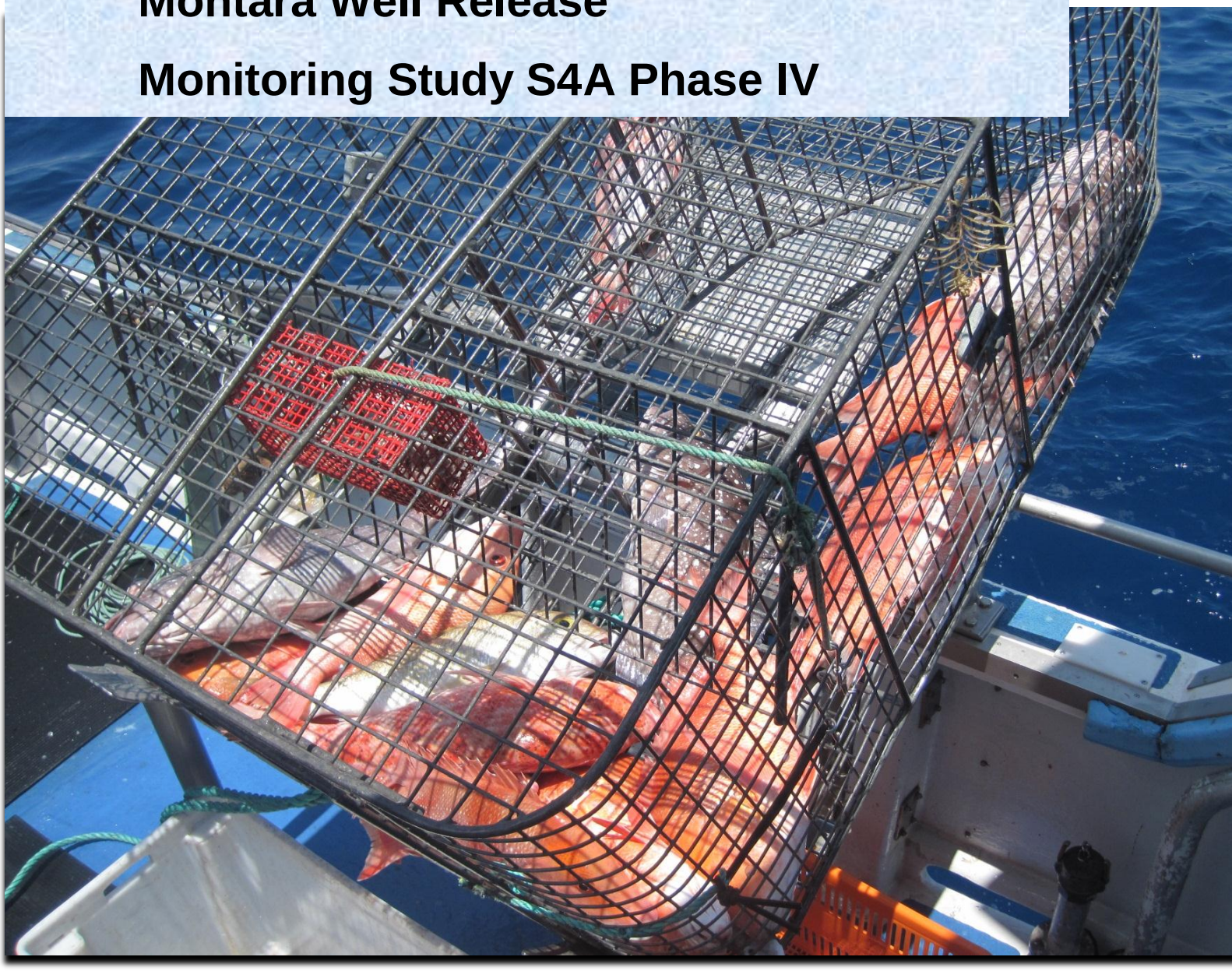
# Montara Well Release

# Monitoring Study S4A Phase IV



## Assessment of Effects on Timor Sea Fish

**Final Report**

Curtin University

# Summary of Results

- Phase IV of the monitoring study conducted two years following the Montara well release collected biological parameters from 486 goldband snapper (*Pristipomoides multidens*) and 395 red emperor (*Lutjanus sebae*) collected over 13 sampling locations.

- For both species of fish, all individuals appeared in good physical condition.

- Goldband snapper were of similar body length at all sites. Red emperor collected close to the well head were smaller in size relative to those from other sites, most likely due to the fishing pressure which this local fish population has experienced in the past two years.

- For both species of fish, individuals collected close to the wellhead had larger livers relative to body size, compared to fish collected at the reference location.

- Biomarkers of exposure to petroleum hydrocarbons showed that in the fish collected close to the well head, no recent exposure had occurred in the recent weeks prior to the sampling.

- As in the past samplings, fish collected within the Heywood Shoal area exhibited elevated biomarker responses indicating recent exposure to petroleum hydrocarbons. Fish returning positive biomarker responses were collected at a site close to the Cornea natural seep.

- Maturation of gonadal tissues was progressing in similar stages at impacted and reference sites.

- Overall, fish collected at the four sites closest to the rig, all located within 27 NM from the well head, showed no recent exposure to petroleum hydrocarbons. Biochemical markers measured in fish collected close to the well head showed that biomarkers of exposure to petroleum hydrocarbons measured in individuals collected in the area most impacted by the Montara well release have returned to reference levels. The liver size relative to body size in the fish collected close to the well head was larger compared to fish originating from a reference site however, this could be related to local nutrient enrichment, or to past exposure to petroleum hydrocarbons.

- Future monitoring will indicate if liver somatic index in fish collected close to the well head shows a trend towards return to reference conditions, or if the observation results in a permanent condition related to natural environmental conditions present at these sites.

- Phase IV of the monitoring program has provided valuable baseline data for future monitoring of produced formation water discharge when operations commence at the offshore facility.

# Preface

This report was prepared by Marthe Monique Gagnon and Christopher Rawson from the Department of Environment and Agriculture, Curtin University. The sampling event recorded herein occurred aboard the FV Megan M between the 16th of October and the 7th November 2011.

# Acknowledgements

Special thanks to the skipper (Grant Barker) and crew (Lindsay Smith and Mitch Seelander) of the FV Megan M for their assistance in the collection of fish samples. Thanks to Tomoe Ota for the careful processing and examinations of gonadal tissues.

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| B | 22 Aug 2012 | Comments provided | | G. Nicholson* |
| C | 14 Sept 2012 | Final Report submitted to PTTEP | MM Gagnon<br>CA Rawson | |

* See Appendix I for Comments and Author's responses

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# Disclaimer

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# Executive Summary

Curtin University was contracted by PTTEP Australasia to conduct a fish monitoring study on demersal and pelagic fishes in the Timor Sea, in waters affected by the Montara well release. In November 2011 Associate Professor Marthe Monique Gagnon and Dr. Christopher Rawson conducted 3 weeks of field sampling aboard the FV Megan M commercial fishing vessel, collecting biopsies of serum, liver, gonadal tissue and bile for laboratory biomarker analysis. This study was the fourth such study following the well release and utilised the same sites as earlier phases of the program with 5 additional sites to the north-west and south east of the Montara well head.

The sampling event targeted the commercially important demersal species goldband snapper (*Pristipomoides multidens*) and red emperor (*Lutjanus sebae*). A total of 881 demersal fish were captured during the study with biopsies taken on 519 fish.

The selected physiological indicators included the condition factor, liver somatic index, and gonado-somatic index. Biochemical markers selected for their relevance to exposure to petroleum hydrocarbons were liver detoxification enzymes, biliary polycyclic aromatic hydrocarbon metabolites, liver integrity, and oxidative DNA damage. In addition, histological examination of the gonads was performed.

Previous studies conducted in the areas affected by the Montara incident showed that one year following the hydrocarbon well release, physiological parameters as well as biochemical markers of fish health showed a return towards reference levels for fish captured close to the rig location, with the exception of liver size which varied between impacted and reference areas, and a few fish collected at one impacted site (Heywood Shoal) which showed continuing evidences of exposure to petroleum hydrocarbons.

Phase IV of the study was conducted 24 months following the end of the hydrocarbon release. Goldband snapper collected at the sites closest to the rig were of similar size to those originating from a reference site, while red emperor collected close to the rig were of smaller size relative to their counterpart collected from a reference site. While goldband snapper has a relatively rapid growth rate and did not show population size differences, red emperor is a slow growing fish. This smaller size observed in red emperor collected close to the rig is likely due to the recent fishing pressure experienced by the population at this location, combined with the slow growth rate of this fish species. While the physical condition of the fish of both species was good at all sites, the liver size relative to body size of both species of fish remained elevated at the sites closest to the rig. However, other biomarkers of exposure such as liver detoxification enzymes, PAH biliary metabolites and oxidative DNA damage were comparable in fish collected close to the rig and in fish originating from the reference site, confirming that uptake of petroleum hydrocarbons has not occurred in the recent weeks. For male and female individuals of both species, the gonad size relative to body size of adult fish collected close to the rig were similar to those collected at the reference site as were the stage of egg maturation within the female gonads.

Of particular interest were the fish collected in the vicinity of Heywood Shoal. In previous studies a few individuals returned positive biomarkers of exposure to petroleum hydrocarbons, suggesting the possibility of exposure to natural seepages in the area. In Phase IV, four locations in the vicinity of Heywood Shoal were discretely sampled to identify if these biomarkers of exposure were still high two years following the incident. Male red emperor exhibited a significant liver detoxification activity at one of the sites, suggestive of exposure to hydrocarbons. Female red emperor from the same site had liver detoxification activity similar to those fish from the reference area (female fish frequently have hormonally-inhibited liver detoxification systems). Red emperor collected at the three other sites close to Heywood Shoal, as well as all collected goldband snapper, had liver detoxification activity similar to those fish originating from the reference site. Biomarker

induction in male red emperor collected in the area of Heywood Shoal is consistent with past observations made at this site. It has been noted that the Cornea seep is in proximity to one of the Heywood Shoal sampling sites, and might contribute to the ongoing high biomarker responses observed at Heywood Shoal. .

# Table of Contents

# List of Figures

# List of Tables

# Background

The Montara incident which commenced on Friday 21st August, 2009, resulted over 74 days in the release of gas, condensate and crude oil into the Timor Sea and released an estimated 23,000 barrels of oil and gas condensate into the Timor Sea. The release occurred in an area utilised for commercial fishing activities (Northern Demersal Scale Fishery). In addition, the area is an ecologically important habitat to a variety of marine life including fish, reptiles, cetaceans and aquatic birds. Short- and long-term impacts are possible following exposure to petroleum hydrocarbons, and consequently it is imperative that any impact of the well release on commercial fish populations be assessed.

The most relevant and sensitive assessment of petroleum hydrocarbon exposure and impact on fishes is through the use of biochemical markers (biomarkers) of fish health – biomarkers are sublethal biological endpoints that can be used to quantify exposure or effect. No single biomarker can give an overall assessment of fish health and consequently, a suite of biomarkers is measured on each individual fish. These include measuring the metabolites of polycyclic aromatic hydrocarbons (PAHs) in the bile of the fish (PAHs are generally not accumulated in fish muscle; Varanasi et al., 1989), estimating the amount of DNA damage that has occurred, measuring the activity of liver detoxification enzymes, and measuring liver integrity. In addition, histological examination of the gonads can give important information on alteration to the reproductive state of the animal as a result of exposure to the contaminants.

Immediately following the control of the 2009 well release, Curtin University was contracted by PTTEP Australasia on agreement with the Australian Government Department of Environment Heritage, Water and the Arts (DEWHA) (now SEWPaC) to investigate (using biomarkers of fish health) any exposure and/or effects of the well release on fish health in the Timor Sea. As part of the scientific monitoring program agreed to by DEWHA two assessments would initially be made to investigate these exposures and effects and note any changes over the intervening time (Phase I and Phase II). Samples comprising these assessments were collected in November 2009 and March 2010. Following these initial sampling events a further study (Phase III) was conducted 12 months following the end of the hydrocarbon release. Shortly after the end of the incident, these studies investigating fish health showed evidences of exposure to petroleum hydrocarbons at sites close to the West Atlas drilling rig, increased liver size and increased oxidative DNA damage. One year after the end of the well release however, a reduced biomarker response suggested an ongoing trend towards a return of biomarkers to reference levels (Gagnon and Rawson, 2011).

The current study was undertaken as a follow-up to Phase I-III investigations. It collected samples at 13 sampling sites located between 1 NM and 110 NM from the West Atlas drill rig. It also performed intensive sampling at one specific location, Heywood Shoal, as previous investigations showed that a few individual fish collected at this site returned positive results suggesting continuing exposure to petroleum hydrocarbons.

# Project Aims (Phase IV)

- To further characterise any exposure, including the geographical extent of the exposure, of commercially important Timor Sea demersal fishes to petroleum hydrocarbons following the Montara well release.
- To evaluate if fish health, including reproductive health, is currently affected by exposure to petroleum hydrocarbons.
- By re-sampling the same sites as investigated in Phases I - III of the monitoring program, to investigate any long-term trends in physiological indices and biomarker levels in commercially important fish species from impacted and reference areas.
- To expand the number of sites studied to gain a more complete understanding of the biochemical and health status of fish in the Timor Sea, especially at Heywood Shoal.
- To establish baseline data for future monitoring following the commencement of PFW discharge at the Montara wellhead.

# Sampling Timeline

| | |
|---|---|
| 16th October 2011 | Christopher Rawson arrives Darwin (PM). |
| 17th October 2011 | Christopher Rawson arranges delivery of field gear from PTTEP depot to Megan M. 1300hrs: Monique Gagnon arrives Darwin. |
| 18th October 2011 | 1900 hrs: Megan M departs from Fishermans Wharf, Darwin. Aboard: Scientific team: Monique Gagnon and Christopher Rawson (Curtin University); FV Megan M skipper Grant Barker; FV Megan M crew Lindsay Smith, Mitchell Seelander. Steam due west. |
| 19th October 2011 | Continue steaming toward sampling area. Organisation of field gear in preparation for scientific sampling. |
| 20th October 2011 | 1200hrs: Arrive Site 8. Traps set. 1600hrs: traps lifted and scientific sampling commences. Anchor overnight at Site 8 with traps set for red emperor. |
| 21st October 2011 | 0600hrs: Traps lifted (full complement of fish captured). 1200hrs steam toward Site 9. 1400hrs: Traps set at Site 9. 1700hrs: traps lifted and reset for overnight soak. |
| 22nd October 2011 | 0630hrs: Overnight traps lifted and reset. 1200hrs: traps lifted. (full complement of fish captured). Steam to Site 15. 2300hrs: arrive Site 15 and set anchor. |
| 23rd October 2011 | 0600hrs: traps set (Site 15). 1100hrs: traps lifted and reset. 1400hrs: traps lifted. 1700 steam SE to Site 14. 1930hrs: arrive Site 14 and set anchor. |

| | |
|---|---|
| 24th October 2011 | 0600hrs: Traps set (Site 14). 1100hrs: Traps lifted and reset. 1400hrs: Traps lifted. Steam toward Site 2. 1930hrs: Arrive Site 2 and set anchor. |
| 25th October 2011 | 0600hrs: Traps set (Site 2) within 2 NM of Montara well head. 1130hrs: traps lifted and reset. 1930hrs: sampling completed. Steam to Site 1. |
| 26th October 2011 | 0630hrs: Traps set (Site 1). 1130hrs: traps lifted and reset. 1615hrs: traps lifted (full complement of fish collected). 1800hrs: steam toward Site 13. 2300hrs: Arrive at Site 13 and set anchor. |
| 27th October 2011 | 0550hrs: traps set (Site 13). 1100hrs: traps lifted. Only goldband snapper collected at this site (designated 13A) so selected site for red emperor 12NM NE (designated Site 13B). 1230hrs: set traps (Site 13B). 1530 hrs: traps lifted. 1800 hrs: Steam toward Site 12. |
| 28th October 2011 | 0600hrs: traps set (Site 12). 1100hrs: traps lifted and reset. 1600hrs: traps lifted and steam toward Site 4. |
| 29th October 2011 | 0530hrs: traps set (Site 4 – Heywood Shoal). 1030hrs: traps lifted and reset. 1500hrs: traps lifted and reset. 1800: traps lifted and reset. 1900hrs: steam toward Site 11. |
| 30th October 2011 | 0630hrs: traps set (Site 10). 1130hrs: traps lifted and reset. 1700hrs: traps lifted (full complement of fish captured) and steam toward Site 11. |
| 31st October 2011 | 0600hrs: traps set (Site 11). 1200hrs: traps lifted and reset. 1730hrs: traps lifted and steam toward Site 16. 1930: Arrive Site 16 and set anchor. |
| 1st November 2011 | 0515hrs: traps set (Site 16). 1030hrs: traps lifted and reset. 1630hrs: traps lifted and steam toward Site 5 (Browse Is). |
| 2nd November 2011 | 0130hrs: arrive Browse Is. and set anchor. 0500hrs: traps set (Site 5). 1100hrs: traps lifted and reset. 1530hrs: traps lifted. Completion of scientific sampling. Steam toward Darwin. |
| 3rd November 2011 | Steaming toward Darwin. |
| 4th November 2011 | Steaming toward Darwin. |
| 5th November 2011 | Steaming toward Darwin. 1400hrs: arrive Darwin Harbour. 1900hrs: Enter Duckpond through tide lock and disembark FV Megan M. Equipment readied for transport to Perth via PTTEPAA (TNT). |

| | |
|---|---|
| 6th November 2010 | 0900hrs: Field gear picked up by PTTEPAA (Steve Welch). Cryogenic dry shippers refilled. Frozen samples sent via cold transport. |
| 7th November 2011 | Monique Gagnon and Christopher Rawson depart Darwin and arrive Perth. |

# Sampling Sites

The sites from which fish were collected were based on those sampled during Phases I – III with the addition of extra sites at the request of PTTEP and the deletion of other sites where only pelagic fishes were previously sampled. When specific sites previously fished were known to produce either lower catches or smaller fish, traps were generally set at locations slightly removed from those previously sampled as part of the Montara monitoring program. The locations of the original sampling sites were selected based on the information on the extent, location and direction of hydrocarbon sheen and/or surface (wax) residue (based on AMSA satellite imagery and ship and aircraft observations) provided by DEWHA and PTTEP Australasia at the time of the S4A Phase I. Based on information available reference sites were selected not less than 80 NM south west of the West Atlas drilling platform. Information provided subsequent to the Phase I sampling suggested that the well release had affected areas outside of this radius. Further, sites were selected based on water depth (shallow reef <30m, or deep sea > 70m) and sea floor structure (hard or soft bottom) which allowed for the reasonable expectation of consistency of catch diversity and numbers (Figure 1, Table 1).

- Site 1 located 20 NM to the SSW of the West Atlas drilling rig with Site 2 located within 2 NM as in previous phases of the study.
- Site 3 was selected for the sampling of pelagic species during Phase I of the study. This site was deleted in subsequent sampling events since insufficient numbers were captured after an extensive sampling effort.

- Site 4 was located at Heywood Shoal to the SW of the Montara well head and has been sampled in all previous sampling events. As a result of previous results sampling was intensified at this location, with 3 extra sites (Sites 10, 11 and 16) added to better characterise potential hydrocarbon exposure at this location.
- Sites 5, 6 and 7 were located within the reference area. Sites 6 (Echuca Shoal) and 7 (Scott Reef) were not required in Phase IV as only pelagic fishes were captured at these sites. In Phase IV, demersal fishes were captured at Site 5 (Browse Island). This site represents the reference site for demersal fish.
- Sites 8 and 9 were designated as reference sites during a sampling trip conducted by WA Fisheries in January 2010 and were included in Phases II, III and IV of study S4A. Examination of satellite imagery however later revealed that these sites were also impacted by the hydrocarbon slick.
- Sites 12, 13A and 13B to the SE of the Montara well head were added to the sampling program for Phase IV at the request of PTTEP. These new sites, along with the existing reference site, will provide background data for future monitoring once the new production facility is operational.
- Sites 14 and 15 were located to the NW of the Montara well head and were added in Phase IV of the study. These sites were located in 70 – 120m water and were sampled for demersal species only.

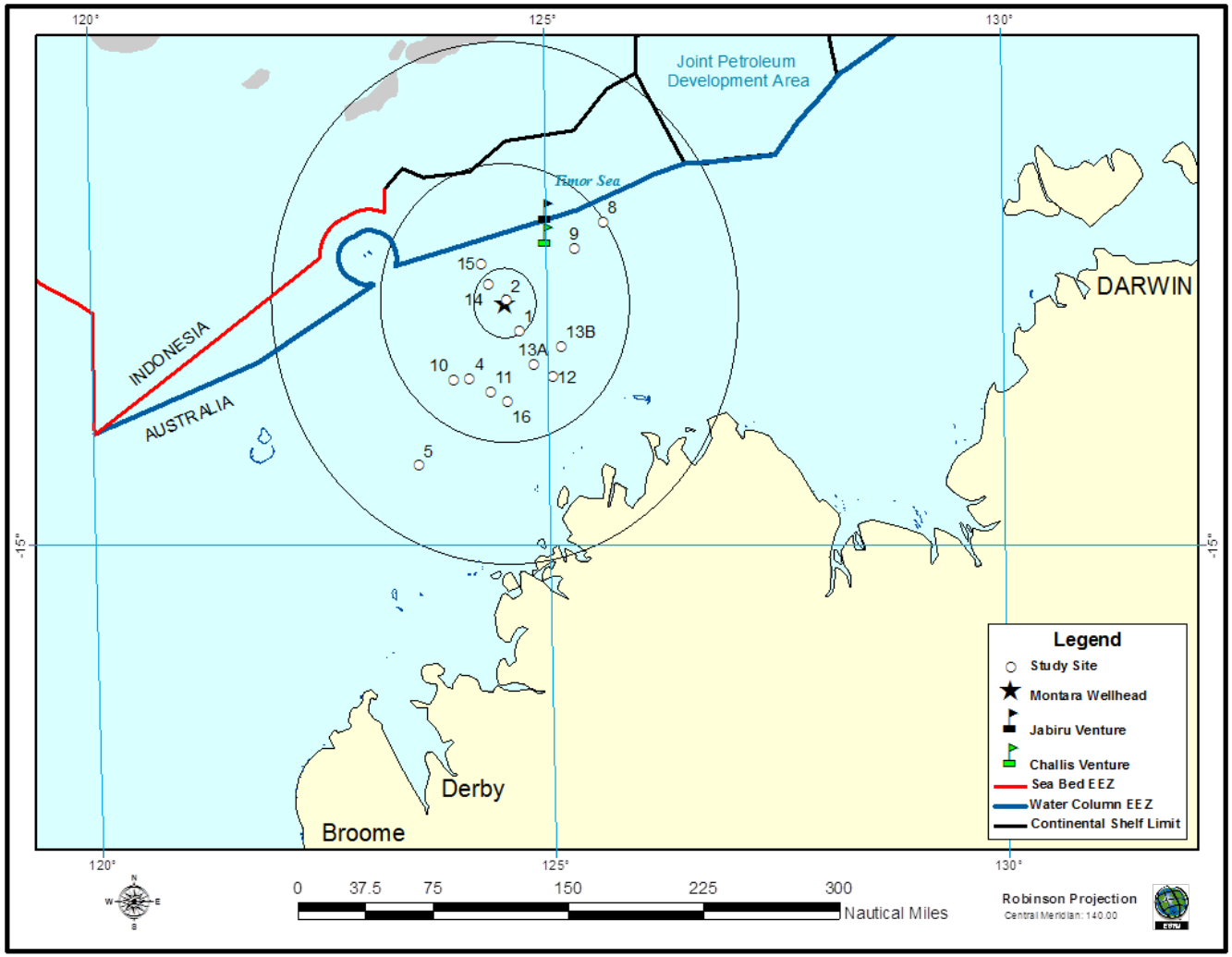



**Figure 1. Map showing the location of the Montara well head and the sampling sites. Concentric rings represent 20, 80 and 150 NM from the well head.**

**Table 1. Details of S4A Phase IV sampling sites**

| Site | | Location | | Distance from West Atlas |
|---|---|---|---|---|
| | | Lat (°) | Long (°) | (NM) |
| 1 | | 12.9450 S | 124.900 E | 19 |
| 2 | | 12.6545 S | 124.5575 E | 1 |
| 4 | Heywood Shoal | 13.4090 S | 124.1437 E | 50 |
| 5 | Browse Island | 14.2422 S | 123.5692 E | 110 |
| 8 | WA Fish. Ref | 11.9016 S | 125.6345 E | 80 |
| 9 | WA Fish Ref | 12.1505 S | 125.3120 E | 55 |
| 10 | | 13.4207 S | 123.9722 E | 56 |
| 11 | | 13.5303 S | 124.6375 E | 52 |
| 12 | | 13.3912 S | 125.0572 E | 53 |
| 13A | | 13.2712 S | 124.8492 E | 40 |
| 13B | | 13.0985 S | 125.1492 E | 44 |
| 14 | | 12.5017 S | 124.3632 E | 15 |
| 15 | | 12.3003 S | 124.2841 E | 27 |
| 16 | | 13.6228 S | 124.5522 E | 57 |

# Fish Sampling

The methods of fish capture were similar to those in S4A Phases I, II and III. Demersal fishes were collected from 70-100m depth using baited stainless steel fish traps (Figure 2). The traps were dropped at the sampling locations and left for between 1 and 12 hours. Two demersal species were targeted: goldband snapper (*Pristipomoides multidens)* and red emperor (*Lutjanus sebae*).

## Morphology

Each fish selected for analysis was initially assessed for length (using a mm scale ruler) and for weight using an electronic spring balance. Each fish was examined for external abnormalities including lesions or excessive fin damage.

**Figure 2. Method of demersal fish capture. Baited steel fish trap prior to deployment. In picture: Matthew Badart (left) and Beau Pieterman (right).**

## Biopsies Collected

Fish selected for biopsy (approximately 20 of each target species from each site) were sacrificed by *iki jimi* (spike through the brain) and a vacuutainer and needle were used to collect blood from the caudal vein. These blood samples were allowed to coagulate and then centrifuged 2400 x *g* for 10 mins and the serum supernatant immediately frozen in a cryogenic dry shipper (approx. -190°C). The fish was then dissected along the ventral line and inspected internally. Many of the fish were significantly infested with parasites in the body cavity. In red emperor there were mainly nematodes while in goldband snapper most of the parasites were encapsulated and adhesive to body organs (liver, gonads, digestive tract). The following biopsies were collected:

1. Serum samples (see above);
2. Bile was collected from the gall bladder using a 1 ml syringe and frozen at -20°C;

3. The liver was removed, weighed and subsamples placed in cryogenic vials and immediately frozen in liquid nitrogen for later analysis;
4. The gonads were removed and weighed. Where available the gonads of 5 male and 5 female fish of each species from each site were preserved in glutaraldehyde for histology.

## Gonad Samples for Histology

At each study site (except Site 16), gonads were collected from 5 male and 5 female individuals of each species (230 samples total). No samples were collected at site 16 due to several additional sites, including site 16, being sampled during the trip, and all extra preservation vials and solutions were consumed. The samples have been processed at Curtin University, Ecotoxicology laboratories.

## Additional Sampling

### Stomach contents

Stomach and intestinal contents were collected from 10 of each species at each location where available. The stomach contents of the demersal fish were biased due to the bait placed in the cages. Consequently, the stomach contents was collected for demersals only if it was identified as 'other than bait', The more relevant intestine contents of the demersal fish, if any, was collected when the stomach was empty. In total 117 stomach/intestinal content samples were collected.

### Flesh Samples

White muscle samples were collected from 10 fish per species per site, and frozen at - 20ºC

## Additional Samples

Samples taken in addition to biopsies for analysis at Curtin University were collected from impacted and reference fishes. Gut contents were collected for 10 goldband snapper and 10 red emperor at each of the study sites (260 samples total). These samples are being held at Curtin University until chemical analysis is requested. Samples of white muscle were also collected from 20 individuals of each species

from each sites. These samples will be stored at Curtin University for one year, should PTTEP opt for additional chemical or olfactory taint assessment.

# Laboratory Methods

## Physiological Parameters

The condition factor (*k*) of the fish was calculated as:

$$k = \left[\frac{W_g}{{L_f}^3}\right] \times 10^6$$

where $W_g$ is the gutted weight of the fish and $L_f$ is the fork length. Condition factor gives an indication of the health status, or 'fattiness' of the animal. The liver somatic index was calculated as

$$LSI = \left[\frac{W_L}{W_g}\right] \times 100$$

where $W_L$ is the liver weight, and gives an indication of the size of the liver relative to the body size. Similarly the gonado-somatic index (GSI) was calculated as:

$$GSI = \left[\frac{W_G}{W_g}\right] \times 100$$

where $W_G$ is the weight of the gonad. GSI is a measure of the fish's reproductive investment.

## Liver Detoxification Enzymes (EROD activity)

In the laboratory, a liver homogenate is centrifuged to isolate the microsomes containing the detoxification enzymes. These microsomes are then required to metabolise a model contaminant: ethoxyresorufin. The enzyme metabolising ethoxyresorufin is therefore named 'ethoxyresorufin-*O*-deethylase' (EROD). The metabolism of ethoxyresorufin by EROD enzyme activity results in a fluorescent product, resorufin, which can be measured by fluorimetry at excitation wavelength 530 nm and emission wavelength 585 nm (Hodson *et al.*, 1991). A higher fluorescence indicates an increased abundance of enzyme that resulted from the fish assimilating and metabolising high levels of contaminants. The enzyme activity is reported by unit of protein in the isolated microsome fraction, which normalizes the data according to the protein density in the liver tissue.

## Biliary Metabolites

After being processed by liver detoxification enzymes, metabolites are predominantly eliminated via the bile. However, the bile turnover and by consequence the concentration of biliary metabolites is influenced by food intake. Therefore, this biomarker is relevant for recent (weeks) exposure to xenobiotics. The bile of the fish was collected using a 1 ml syringe, and immediately frozen in liquid nitrogen until biliary metabolites determination by fixed-wavelength fluorescence (FF) measurement (Lin *et al.,* 1996). The method is semi-quantitative, and reports metabolised PAHs as 'type of metabolites'. The expression 'type of metabolites' refers to the various aromatic compounds, most occurring as conjugated metabolites of PAHs, that are measured in the bile at naphthalene-, pyrene-, or benzo(a)pyrene-(B(a)P-) specific excitation/emission wavelengths. Fluorescent readings were performed for naphthalene-type metabolites at excitation/emission 290/335 nm using 1-naphthol (Sigma) as a reference standard. For pyrene-type metabolites and B(a)P-type metabolites, readings were made using 1-hydroxy pyrene (1-OH pyrene) as a reference standard at 340/380 nm and 380/430 nm for pyrene- and B(a)P-type metabolites, respectively. Naphthalene-type metabolites are reported in mg of 1-naphthol fluorescence units equivalent per mg biliary protein, and pyrene- and B(a)P-type metabolites are reported in µg of 1-OH pyrene fluorescence units equivalent per mg biliary protein. Therefore, the biliary metabolite levels observed represent fluorescence-equivalents of PAH metabolites used as standard.

## Sorbitol Dehydrogenase (SDH) activity

A blood collection was performed via the caudal artery using a vacuutainer. The blood was allowed to clot on ice for 15 minutes after which it was centrifuged in a refrigerated centrifuge at 3000 *g* for 10 minutes. The serum was immediately collected and frozen at -80°C until analysis of SDH activity. The quantitation of SDH activity was determined by the reduction in light absorbance at 340 nm over time. SDH activity is reported in milli-International Units (mIU), with one U of an enzyme being defined as the amount of this enzyme which will convert 1 µmol of substrate to product, per minute.

## Gonad Histology

Preserved (glutaraldehyde) male and female gonads were washed and transferred to a 50% ethanol solution for longer term storage. The samples sectioned into 10 mm cross-sections which were further dehydrated by progressively increasing the ethanol concentration over four hours. They were then cleared in xylene for a further three hours before embedding in paraffin wax overnight. These samples were placed in paraffin wax blocks for sectioning (5 µm) using a microtome. These sections were fixed to glass slides and gently heated overnight prior to staining. The slides were stained (Haematoxylin – Eosin) using standard methods (Lillie, 1965). This differential staining procedure allows the identification of relevant structures in the tissues (particularly nuclei and membranes) and the identification of any abnormalities. In gonad tissue it also allows the classification of gametes at different stages of development.

Histological slides of the ovaries of selected Goldband snapper and Red emperor females from each site were examined for the development of oocytes. Photographs of random regions of the slides taken under magnification (x 200) were overlain with a grid. Fifty grid locations were randomly selected for each slide and the number of oocytes at each stage (following the scale described by West 1990 and detailed in Table 2) falling within each grid square was determined. Results are presented as the average percentage appearance of each stage in the ovary.

**Table 2. Scale used for determining oocyte stage in goldband snapper and red emperor.**

| Stage Name | Description | Scale |
|---|---|---|
| **Chromatin nucleolar stage** | Primordial follicle; large nucleus with single large nucleolus; cell surrounded by few squamous follicle cells. | 1 |
| **Perinucleolar stage** | Cytoplasm stains uniformly; multiple nucleuoli appear around nuclear periphery. | 2 |
| **Cortical alveoli formation** | Appearance of "yolk" vesicles forming several peripheral rows; oil droplets accumulate in the cytoplasm. | 3 |
| **Vitellogenic stage** | Yolk proteins in fluid-filled spheres appear | 4 |
| **Ripe (mature) stage** | Nucleus migrates peripherally; yolk granules coalesce and oocyte becomes more transparent; rapid increase in size due to oocyte hydration; | 5 |

## Oxidative DNA Damage (8-oxo-dG Content)

The concentration of the oxidative stress biomarker 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in the serum samples was measured using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Trevigen). Briefly, serum samples were diluted (1:5) with the diluent supplied with the kit and a supplied 8-oxodG standard was diluted to create a standard curve (0.94 – 60 ng $L^{-1}$). These were added to a 96 well microplate which was pre-coated with 8-oxodG with an 8-oxodG monoclonal antibody. After 1 hour incubation the plate was washed 6 times (Bio-Rad plate washer) and a secondary antibody (horseradish peroxidase conjugate) was introduced. After further 1 hour incubation the plate was again washed (6 times) and the plate developed with a trimethylbenzine (TMB) substrate and the absorbance (450 nm) was measured (Bio-Rad plate reader) after addition of an acidic stop solution. The intensity of the colour was inversely related to the amount of 8-oxo-dG in the sample or standard. These were standardised against the protein content of each serum sample (Lowry *et al.,* 1951).

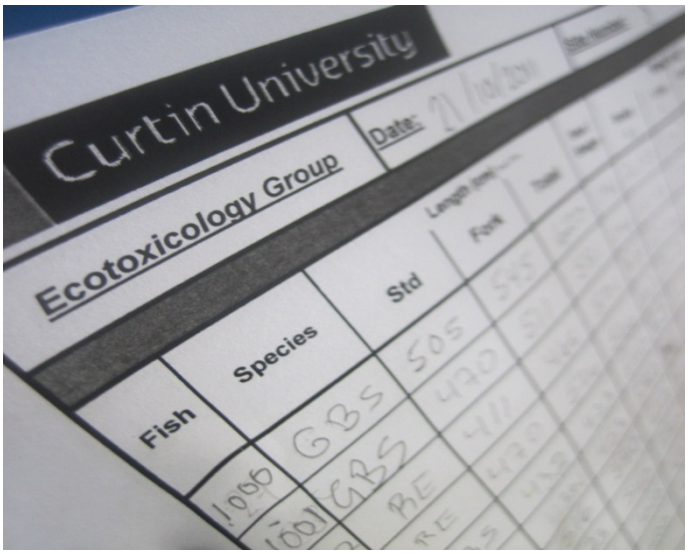
## Statistical Analyses

For each variable measured, descriptive statistics including average and standard errors of the mean have been generated using the program SPSS ver. 20.0. Multiple comparisons focussed mainly on sites closest to the rig (sites 1, 2, 14, 15) relative to the reference Site 5, as other sites were mostly sampled in order establish a baseline for future monitoring of produced formation water discharge. Physiological parameters condition factor (CF), liver somatic index (LSI) and gonado-somatic index

(GSI) as well as biomarker levels were compared using a one-way analysis of variance (ANOVA) after verification of normal data distribution and homoscedasticity. Post-hoc test Tukey's-b was applied to identify specific site differences, if relevant. In cases where data were not normally distributed or heteroscedasticity was observed, non-parametric multiple comparisons were conducted using the Kruskal-Wallis 1-way ANOVA followed by a Mann-Whitney U test. For all statistical tests, a significance level alpha ($\alpha$) of 0.05 was applied.

All graphs are presented as mean ± standard error of the mean (SEM). Number of fish for individual means are listed in Table 2 for physiological parameters CF, LSI, GSI and CF, and according to Table 3 for all biochemical markers except for oxidative DNA damage where N = 10 fish per site.

# Results & Interpretation




## Fish Sampled

A total of 881 fish were sampled across the 13 study sites (486 goldband snapper and 395 red emperor) (Table 3). Male and female fish were captured at each study site with relatively even numbers of both sex collected. Morphological measurements (lengths, weight, liver weight, sex, gonad weight and stage) were collected for each of these fish. There were some juvenile fish for which gonadal sex could not be determined visually.

**Table 3. Numbers of demersal fishes sampled during Phase IV of Study S4A.**

| | | | | Goldband Snapper | | | Red Emperor | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | Site | Male | Fem | Juv | Total | Male | Fem | Juv | Total | |
| Impacted | 1 | 21 | 19 | 0 | 40 | 20 | 20 | 0 | 40 | 80 |
| | 2 | 14 | 15 | 0 | 29 | 17 | 20 | 3 | 40 | 69 |
| | 4 | 11 | 15 | 0 | 26 | 12 | 25 | 2 | 39 | 65 |
| | 8 | 16 | 17 | 0 | 33 | 6 | 11 | 3 | 20 | 53 |
| | 9 | 22 | 17 | 1 | 40 | 10 | 14 | 4 | 28 | 68 |
| | 10 | 29 | 11 | 0 | 40 | 17 | 23 | 0 | 40 | 80 |
| | 11 | 25 | 15 | 0 | 40 | 9 | 14 | 3 | 26 | 66 |
| | 12 | 26 | 15 | 0 | 41 | 8 | 8 | 0 | 16 | 57 |
| | 13 | 26 | 15 | 0 | 41 | 3 | 9 | 1 | 13 | 54 |
| | 14 | 21 | 14 | 2 | 37 | 21 | 18 | 1 | 40 | 77 |
| | 15 | 20 | 19 | 0 | 39 | 15 | 17 | 1 | 33 | 72 |
| | 16 | 17 | 23 | 0 | 40 | 11 | 21 | 2 | 34 | 74 |
| | Total | 210 | 161 | 2 | 373 | 133 | 175 | 13 | 321 | 694 |
| Reference | 5 | 20 | 20 | 0 | 40 | 12 | 14 | 0 | 26 | 66 |
| | Total | 58 | 54 | 1 | 113 | 28 | 39 | 7 | 74 | 187 |
| **Grand Total** | | **268** | **215** | **3** | **486** | **161** | **214** | **20** | **395** | **881** |

# Biopsy Collection

Biopsies for analysis at Curtin University Ecotoxicology laboratories (serum, bile, liver and gonad samples) were collected on a total of 519 fish (263 goldband snapper and 256 red emperor) (Table 4). The quota of 20 goldband snapper for biochemical analysis was reached at all sites but was not reached at 2 sites (12 and 13) for red emperor. It is not expected that this will markedly affect the outcomes of this study

**Table 4. Numbers of fish from which biopsies were collected during the current phase of study S4A.**

| Site | | Goldband Snapper | Red Emperor | Total |
|---|---|---|---|---|
| Impacted | 1 | 20 | 21 | 41 |
| | 2 | 20 | 20 | 40 |
| | 4 | 21 | 20 | 41 |
| | 8 | 20 | 21 | 41 |
| | 9 | 20 | 22 | 42 |
| | 10 | 20 | 21 | 41 |
| | 11 | 20 | 20 | 40 |
| | 12 | 20 | 16 | 36 |
| | 13 | 20 | 13 | 33 |
| | 14 | 21 | 21 | 42 |
| | 15 | 21 | 21 | 42 |
| | 16 | 20 | 20 | 40 |
| | Total | 203 | 193 | 396 |
| Reference | 5 | 20 | 20 | 40 |
| | Total | 60 | 63 | 123 |
| **Grand Total** | | **263** | **256** | **519** |

## General Fish Health

No gross abnormalities or external parasites were observed on any of the specimen of goldband snapper or red emperor collected during Phase IV of the study. Internal parasites were common in both species, with observations of worms adhering to the external walls of the stomach, intestines or liver (Figure 3). The rate of occurrence of parasitic worms, and the abundance of worms in the abdominal cavity, seemed to be similar to those observed in previous samplings.

# Physiological Parameters

Gross indices issued from physiological measurements can be indicative of effects following chronic exposure to pollution. While non-specific to individual contaminants, physiological indices such as condition factor (CF), liver somatic index (LSI) and gonado-somatic index (GSI) represent a first-level screen to identify if effects have occurred, and if further investigations are warrant. In this regard, condition factor is a useful indicator of the 'fattiness' of the fish, may provide information on energy reserves of the fish and possibly the ability of the animal to tolerate toxicant challenges or other environmental stresses. The liver-somatic index might inform on liver pathologies, including liver enlargement upon exposure to chronic contamination. Finally, the gonado-somatic index informs on the reproductive investment made by fish, as reproduction is often one of the first parameter affected in fish exposed to contamination (van der Oost et al. 2003).

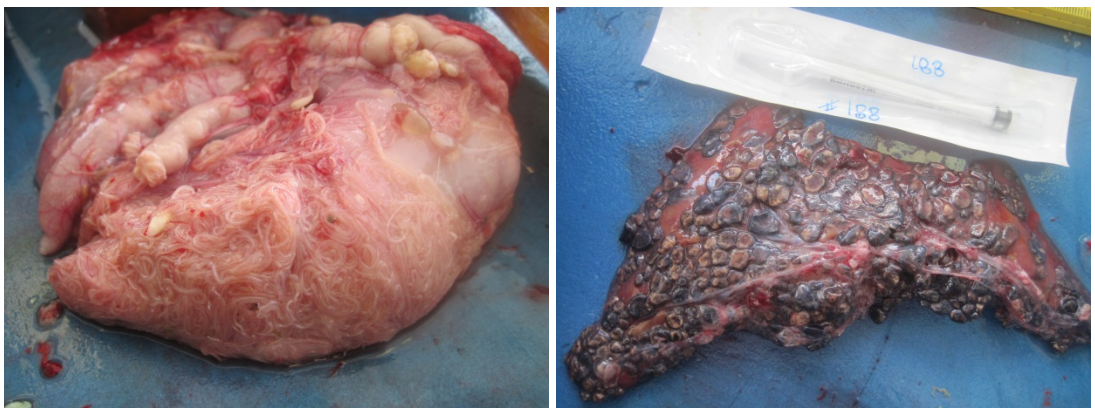


**Figure 3. Parasites commonly found in the abdominal cavity of goldband snapper and red emperor.**

## Condition Factor

The condition factor of goldband snapper collected at three of the 4 sites closest to the rig was similar to the condition factors in fish originating from the reference site (Figure 4). Goldband snapper collected at one site (Site 15) located 27 NM north-west of the rig had higher liver-somatic index relative to reference fish of this species. The very small difference observed in liver size relative to body size might be a result of local environmental conditions found at Site 15 e.g. food abundance, as an altered condition factor is not consistently observed in goldband snapper collected at other sites close to the rig. Moreover, the condition factor of red emperors collected at all sites close to the rig did not either differ from the condition factors in red emperors from the reference site. Condition factor of fish which informs on the general health status and energy reserves of individuals, might be affected by non-pollutant factors such as seasons and nutritional levels (van der Oost et al. 2003). It is therefore concluded that fish of both species collected at the four sites close to the West Atlas rig are in good physical conditions, as measured by their condition factors.

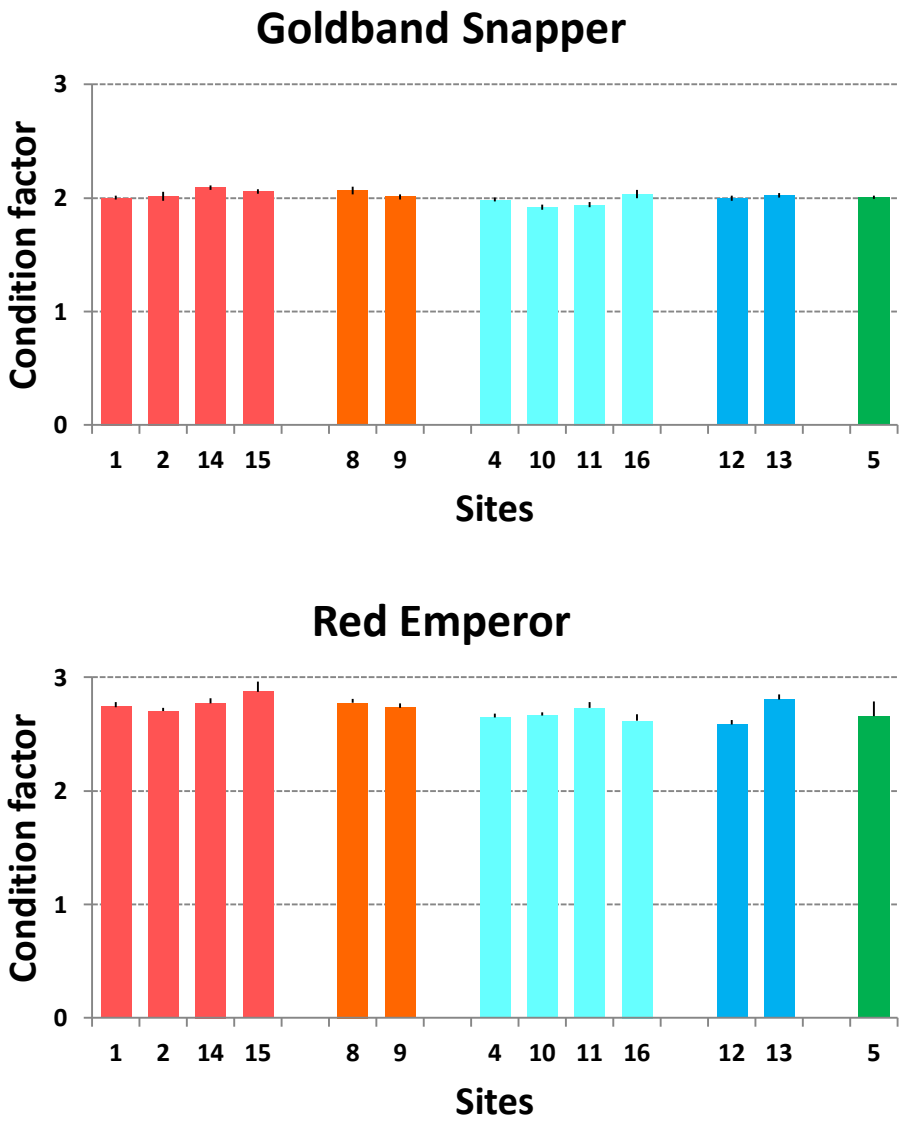



**Figure 4. Condition factors of goldband snapper and red emperors collected at the various study sites during Phase IV of the monitoring study S4A. For both species, fish from all sites had statistically similar CF ($p > 0.05$ in all cases).**

## Liver Somatic Index

The ratio of the liver size relative to body size provides an index that is widely used as a general indicator of chronic exposure to contaminants. A larger liver can be the result of exposure to contaminants, in particular contaminants which are largely metabolised by the liver. However, under field conditions, LSI can also vary according to biotic or abiotic factors other than pollutants, such as onset of reproductive activity.

In goldband snapper collected at three of the sites (sites 1, 2 and 15) located close to the West Atlas well head, the liver size relative to body size was enlarged compared to the goldband collected at the reference site (Figure 5). In red emperor, a similar pattern was observed with fish from all sites located close to the well head having enlarged livers relative to individuals of this species collected from the reference site. Higher liver size relative to body size has been observed for both species of fish in previous samplings of these locations. Findings of the present investigation confirm previous observations (Gagnon and Rawson 2011). While the observation is consistent through time and species, it is unlikely that larger liver sizes are due to continuing exposure to contamination as other biomarkers specific to petroleum hydrocarbons did not confirm exposure of fish to petroleum hydrocarbons at these locations (see 'liver detoxification enzymes' and 'biliary metabolites' below). it is unlikely that enlarged livers are related to reproductive activities, as reproductive status of fish from all sites were similar (see gonado-somatic index, following section).

Enlarged livers in fish collected close to the rig might be related to nutritional status of the fish, in which case increased lipid stores would be deposited in the liver. It is suggested to investigate liver lipid contents of goldband snapper and red emperors in future monitoring.

For both species of fish, LSI has shown altered values in previous post-spill samplings. Liver is a plastic organ with the capability to adjust its mass when sustained metabolic demand occurs and in this regard, the biological response of altering hepatic tissue mass might take several months. Short-term biomarkers of exposure e.g. EROD activity and PAH biliary metabolites are low and suggest no

recent exposure to petroleum hydrocarbons, however it is possible that the liver size has not fully returned to pre-spill conditions in fish collected close to the well head. Alternatively, the lower liver size in fish collected close within 27 NM of the rig might be related to environmental conditions close to the well head, including prey abundance available to the fish. Abundance of preys might not have returned to pre-spill event, resulting in lower energy reserved stored in the liver of goldband snapper and red emperor.

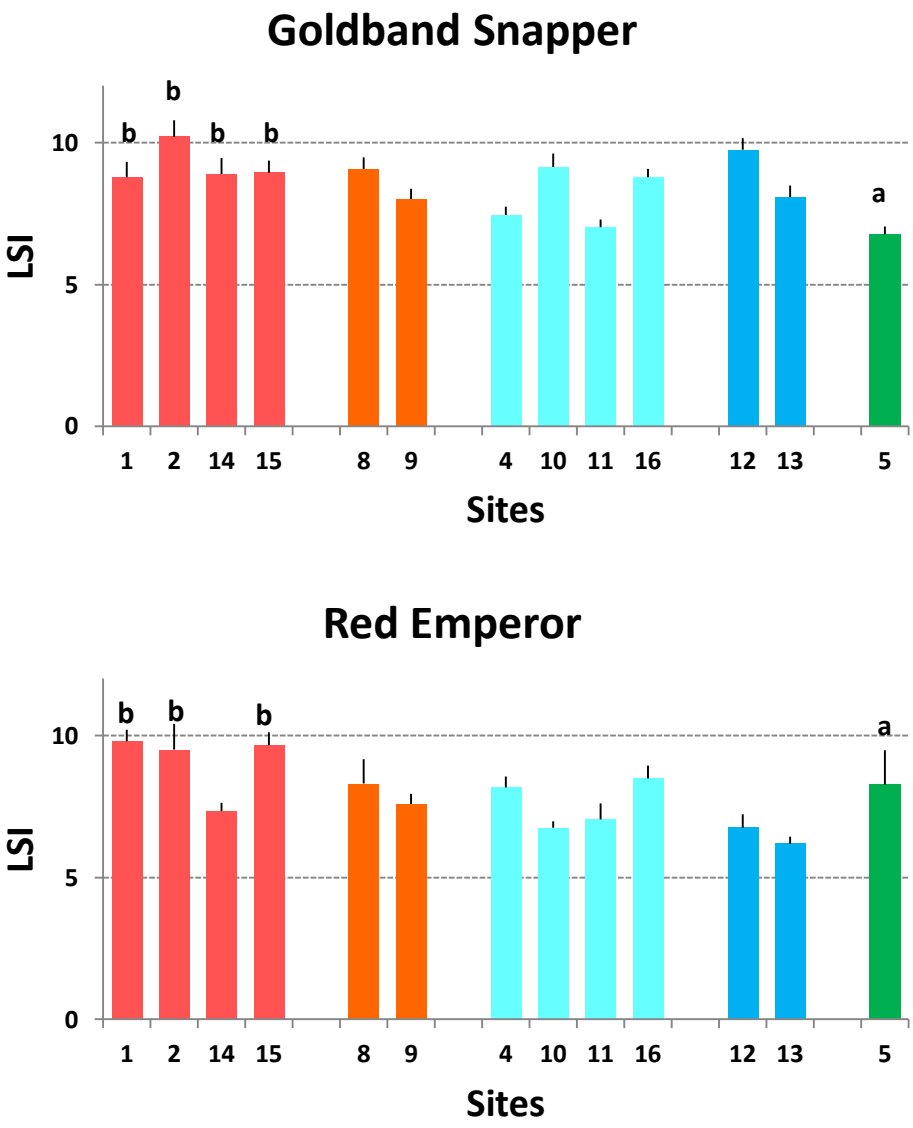



**Figure 5. Liver-somatic index in goldband snapper and red emperors captured at study sites in the Timor Sea in November 2011. Bars with different letters indicate statistical differences. Sites 1, 2, 14, 15 were all located within 27 NM of the hydrocarbon release and were considered impacted, while reference Site 5 was located at 110 NM from the West Atlas rig location. LSI at the sites close to the drill rig was different from LSI in reference fish ($p < 0.000$).**

## Gonado-somatic Index

The GSI, or gonado-somatic index, is a measure of the reproductive competence in fish. There is increasing evidences that chronic, low-level pollution might impair reproduction in fish, leading to a long-term decline and potentially, causing local extinctions of populations (reviewed in Kime, 1995). The gonado-somatic index might be quite variable between populations, but still represents an indication of the reproductive status of the fish.

GSI appeared to vary considerably between sites, especially for female goldband snapper (Figure 6). Differences between sites can be explained by the fact that at most sites, some female fish had well developed mature gonads ready to spawn while other female fish were at earlier phases of gonadal development. At other sites, very few female fish had mature gonads, making the GSI value significantly lower. A lower GSI at some sites does not imply a negative outcome on reproduction, it rather informs on the energy directed by the fish up to the capture date to reproductive maturation of gonadal tissues. To assess if gonad maturation proceeds normally, histological examination of the gonads has been conducted (see next section).Similarly for male goldband snapper, individuals at Site 12 were more advanced in their gonad development relative to fish from the reference site, however, this does not imply a differential reproductive outcome.

For both male and female adult goldband snapper and red emperors, GSI was similar in fish close to the well head (sites 1, 2, 14 and 15) relative to GSI in fish collected at the remote reference Site 5 (Figure 6, Figure 7). The reproductive investment in gonad development appeared to be comparable in all fish from these five locations. This confirms the trend observed in previous monitoring of these sites, where low GSI were initially observed in fish collected close from the well head but were showing a temporal trend towards similar GSI as in the reference fish.

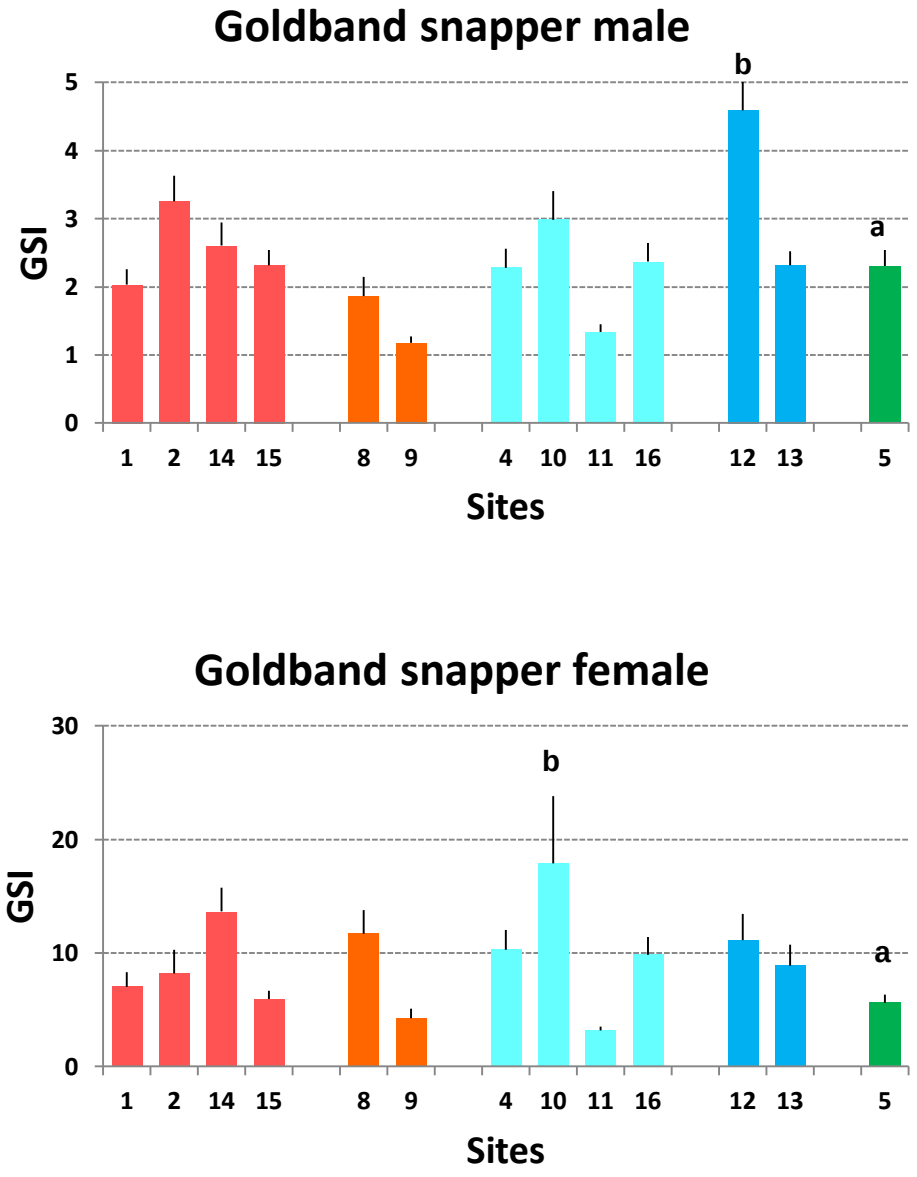



**Figure 6.Gonado-somatic index of male and female goldband snapper captured at study sites in the Timor Sea in Phase IV of the monitoring. Bars with different letters indicate statistical differences. Fish captured at sites within 27 NM of the well head (sites 1, 2, 14, 15) have similar GSI relative to fish from the reference Site 5. GSI in male fish originating from Site 12 is different from GSI in male fish from the reference Site 5 (p =- 0.006). GSI measured in female fish collected at Site 10 is different from GSI measured in female fish from the reference Site 5 (p = 0.001).**

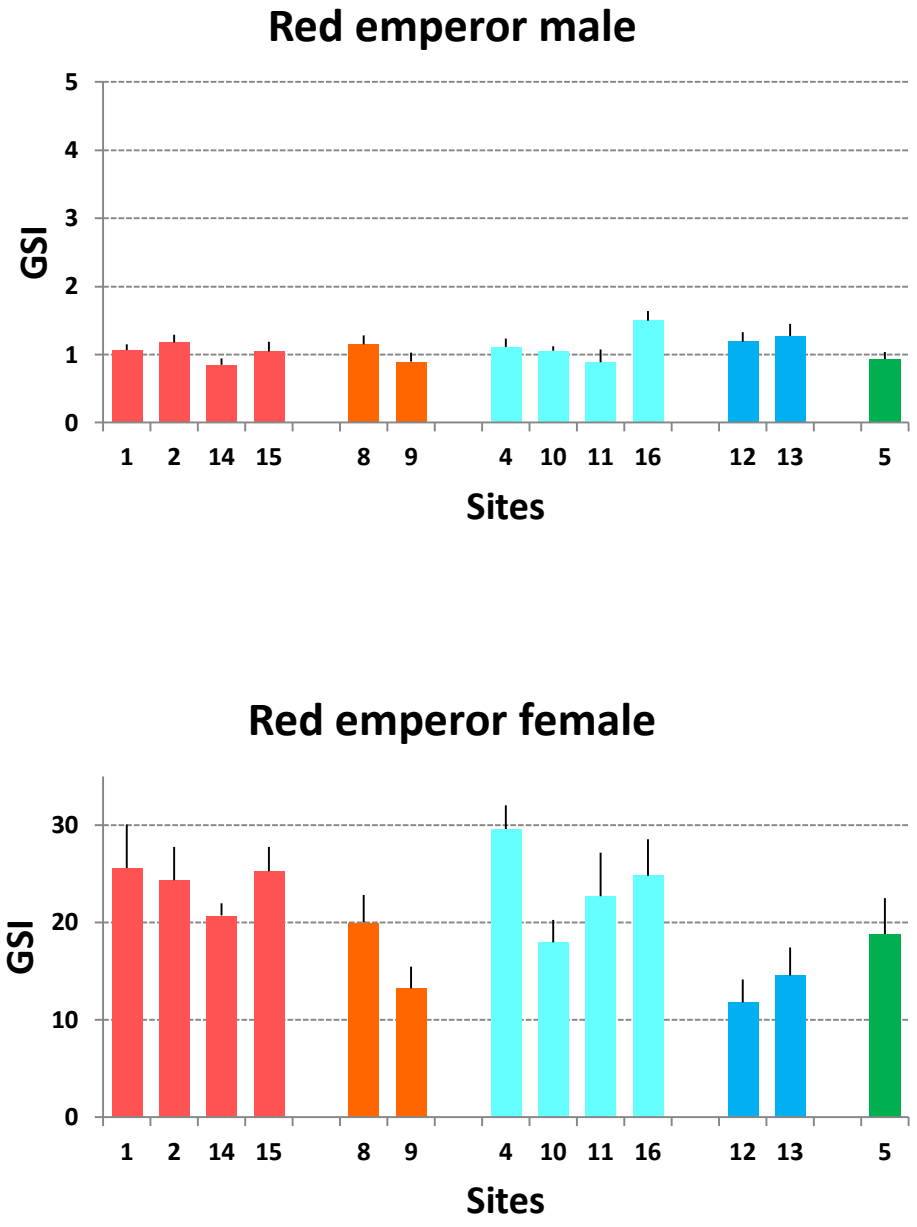



**Figure 7. Gonado-somatic index of male and female red emperors captured at study sites in the Timor Sea in Phase IV of the monitoring. Male and female fish captured at sites within 27 NM of the well head (sites 1, 2, 14, 15) had similar GSI relative to fish from the reference Site 5 ($p > 0.05$ in all cases).**

## Gonad Histology

Histological examination of the male gonads revealed no overall differences between red emperor and goldband snapper at the study sites. Male gonads of each individual contained spermatagonia at a range of developmental stage with the majority having spermatagonia containing fully developed sperm. These fish appeared to be either spawning or about to spawn.

The testes of a single goldband snapper from Site 13 with morphologically male gonads contained ovarian tissue (Figure 8). The testicular tissue was interspersed with oocytes at varying stages of development. While such an occurrence can be due to a contamination event (e.g., Jobling et al 1998), there is no evidence to suggest such a link in this case. The occurrence of simultaneous hermaphroditism (the presence of a male and a female gonad in the same individual) is not unknown in fishes (e.g., some Serranidae). Other species (e.g., some Sparidae) occur as rudimentary hermaphrodites (either having both male and female gonads or gonads with both male and female tissue in early life stages then developing in favour of a single gender with maturity). The occurrence of intersex gonads (the presence of oocytes in a testis) has also been reported as naturally occurring (e.g., Korner et al. 2005). As far as we are aware this is the first time this has been observed in goldband snapper. Throughout study S4A, gonads from over 150 male goldband snapper have been examined histologically and this is the first occurrence of the phenomenon. Given the rarity of this occurrence and the remoteness of the location from any known source of contamination (relative to other sites nearer the Montara well head), it appears that for this species the presence of ovarian tissue in the testes is normal, though uncommon.

The ovaries of female goldband snapper and red emperor examined histologically were without apparent pathology. In general the goldband snapper gonads examined were less well developed than the red emperor with a greater number of individuals lacking stage V oocytes. At sites 15, 11 and 5 none of the goldband snapper examined contained stage V oocytes and at each of the other sites there was at least 1 individual examined with a similar condition (Figure 9).

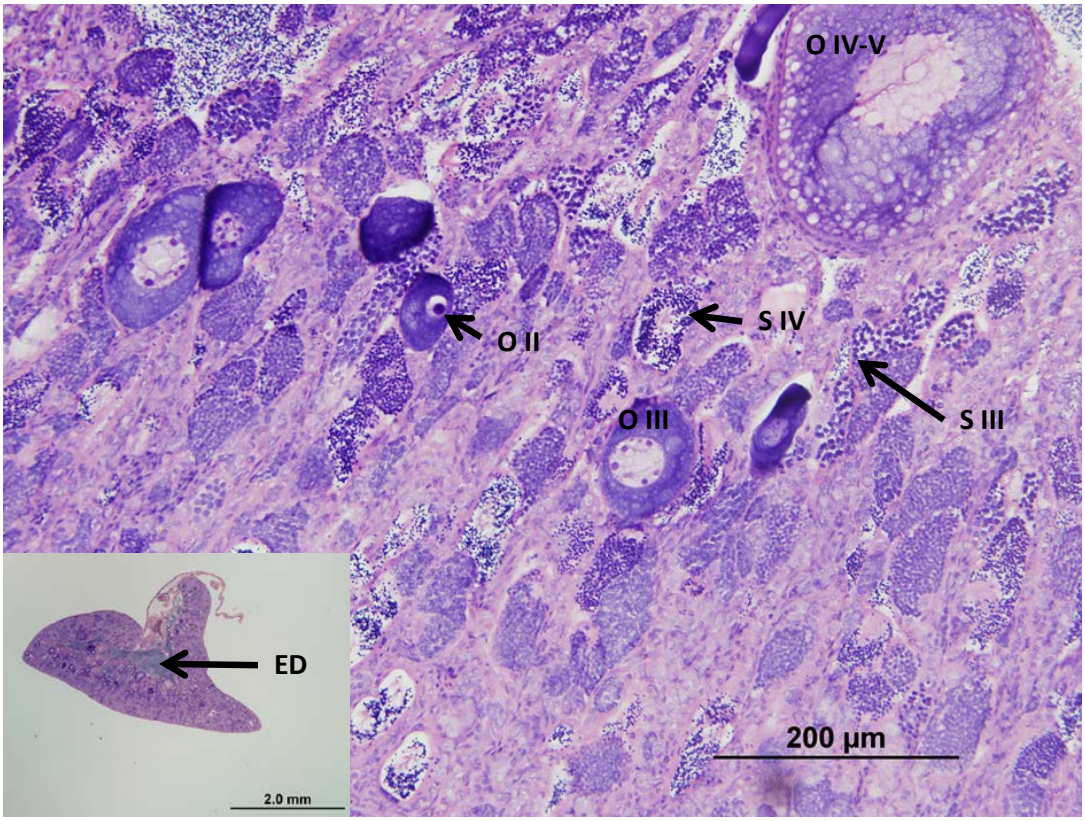



**Figure 8. Histological (HE stain) examination of intersex gonad of morphologically male goldband snapper collected from Site 13. Inset shows characteristic lobed structure of male testis (note the mature sperm in the efferent duct). ED = Efferent Duct, S = Sperm (with developmental stage indicated), O = oocyte (with developmental stage indicated).**

At all sites, there were red emperor, the ovaries from which contained stage V oocytes. This suggests that while there is significant variation within the populations of each species, the red emperor were closer to their optimal spawning time. There was no indicative trend in the presence of stage V gonads associated with the location of the Montara well head. While Site 15 is in close proximity, Sites 11 and 5 are located at a greater distance.

There some between-site variation within each species as to both the dominant oocyte stage present and the furthest oocyte development observed (both are commonly used metrics for determining differences between individuals). The goldband snapper captured in the sites closest to the Montara well head had oocyte stage distributions which were not dissimilar to those at sites further away. Of all the goldband snapper collected, only those from Site 8 had an increased proportion of

Stage I oocytes, indicating that these fish may be later in their reproductive cycle than those at other sites.

Red emperor collected at sites 8 and 9 had an increased proportion of stage I and II oocytes with a decreased proportion of stage III oocytes compared to individuals from other study sites. Sites 8 and 9 were the furthest north east of the Montara well head. This result does not appear related to the Montara release. It is likely a geographically driven difference in spawning time.

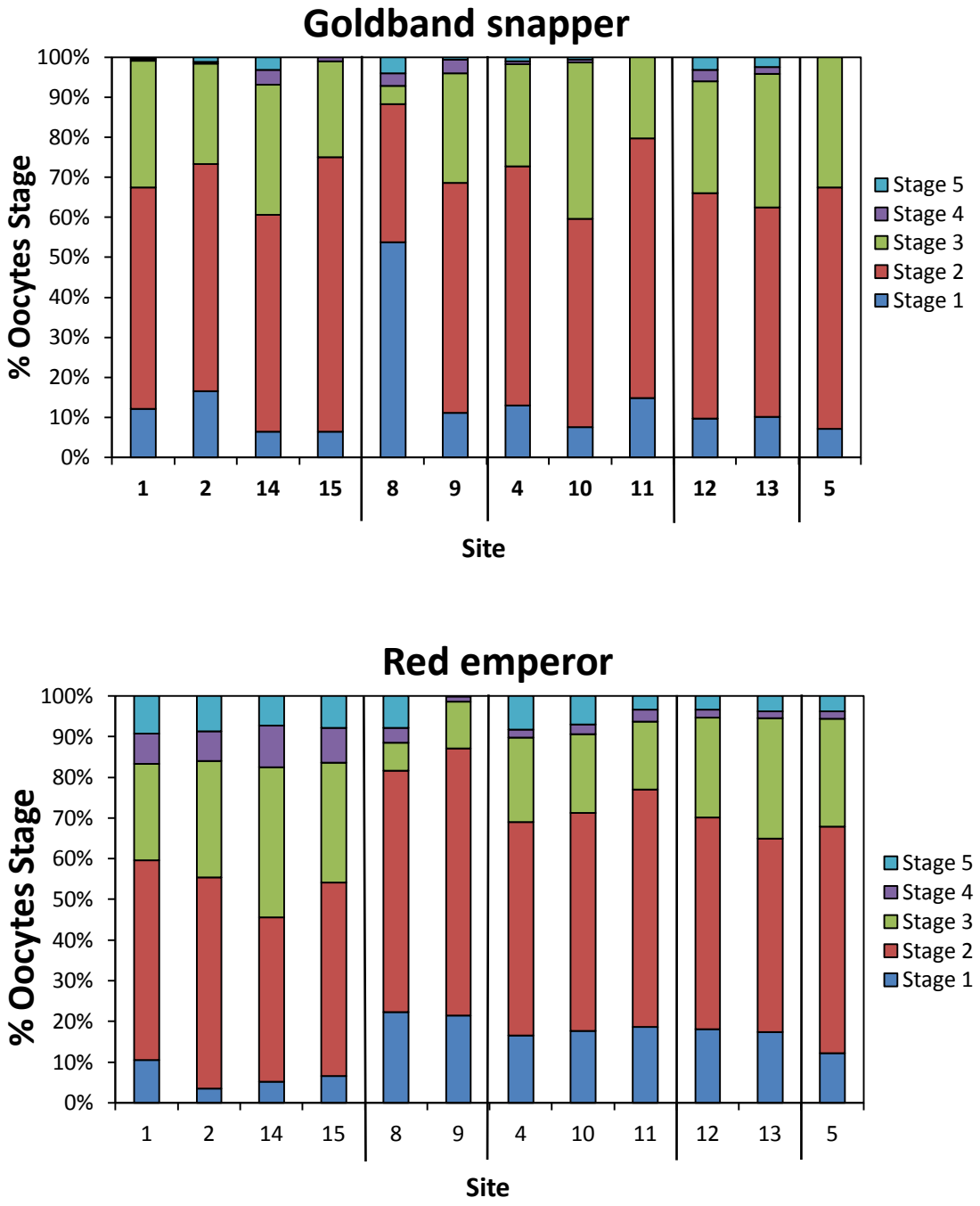



**Figure 9. Percent composition of ovaries of goldband snapper and red emperor from Timor Sea study sites by Oocyte stage. Oocyte stages defined and described given in Table 2.**

# Biochemical Parameters

## Liver Detoxification Enzymes

Liver detoxification enzymes are part of a detoxification system found in most animals. Upon exposure to certain classes of contaminants such as petroleum hydrocarbons, polychlorinated biphenyls, etc., liver enzymes are induced at high levels in order to proceed with oxidation of xenobiotics which aims to eliminate the chemical out of the body. Specific detoxification enzymes, such as ethoxyresorufin-*O*-deethylase (EROD), are measurable in fish livers even if the inducing chemical is well below detectable levels in the environment (Landis and Yu, 1995).

Liver detoxification enzymes are present with relatively low activity in normal animals as these enzymes also fulfil several metabolic functions. In fact, activity of the liver enzymes can vary naturally with sexual hormones, increasing the variability of this biomarker. For example, the presence of estrogen in the reproductively active female fish can significantly inhibit the measured EROD activity levels (van der Oost et al., 2003). Because of this potential alteration by reproductive hormones, liver detoxification activity has to be considered separately for male and female organisms.

Because liver enzymes do perform normal metabolic activities, it is expected to measure background enzymatic activity levels which are specific to each species. Concerns arise when activity levels of enzymes closely correlated to exposure to contaminants e.g. EROD activity, is increased by 2-fold or more in a consistent manner across contaminant-exposed animals.

Previous investigations carried out following the Montara incident revealed that goldband snapper and red emperor collected in close proximity to the West Atlas rig exhibited elevated hepatic EROD activity, suggesting that these fish were exposed to, had assimilated, and were in the process of metabolising petroleum hydrocarbons (Gagnon and Rawson 2011). In Phase IV investigation, liver detoxification activity in both species of fish, and for both sexes, were similar in fish collected close to the well head relative to the EROD activity in fish from the reference area (Figure 10, Figure

11). This result suggests that fish collected in close proximity of the well head are no longer exposed to the inducing compound which triggered EROD activity levels in past studies. It also provides background activity levels and variability for comparative purposed in future studies.

It is noted that male red emperor collected at Site 10 had a significantly higher liver detoxification activity relative to male red emperor from the reference Site 5 ($p < 0.001$). High EROD induction suggests that these fish are exposed to compounds inducing EROD activity, which might originate from natural seepage.
This result supports observations from previous samplings where several individuals collected at Heywood Shoal exhibited high EROD activity and biliary metabolite levels. A similar situation might occur at Site 8.

Similar conclusions were reached when a three-year fish health monitoring study was conducted following the 2002 Prestige oil spill that occurred off the coast of Spain. Biomarkers of fish health measured in the Spanish study included EROD activity, with measured levels returning to baseline levels after two to three years post-spill (Martinez-Gomez et al. 2009). The Prestige oil involved a heavy fuel oil (M-100) of different composition to the Montara oil, however both crude oils had a low content of high molecular weight polycyclic aromatic hydrocarbons (PAHs) (Alzaga et al. 2004). In contract, the cold environment in which the heavy fuel oil from the Braer spill (Shetland Islands, United Kingdom, 1993) was discharged was still a major concern 6 years after the event (McGenity et al. 2012). Generally, crude oils with a low content of high molecular weight PAH are prone to rapid biodegradation in marine tropical waters (McGenity et al. 2012).

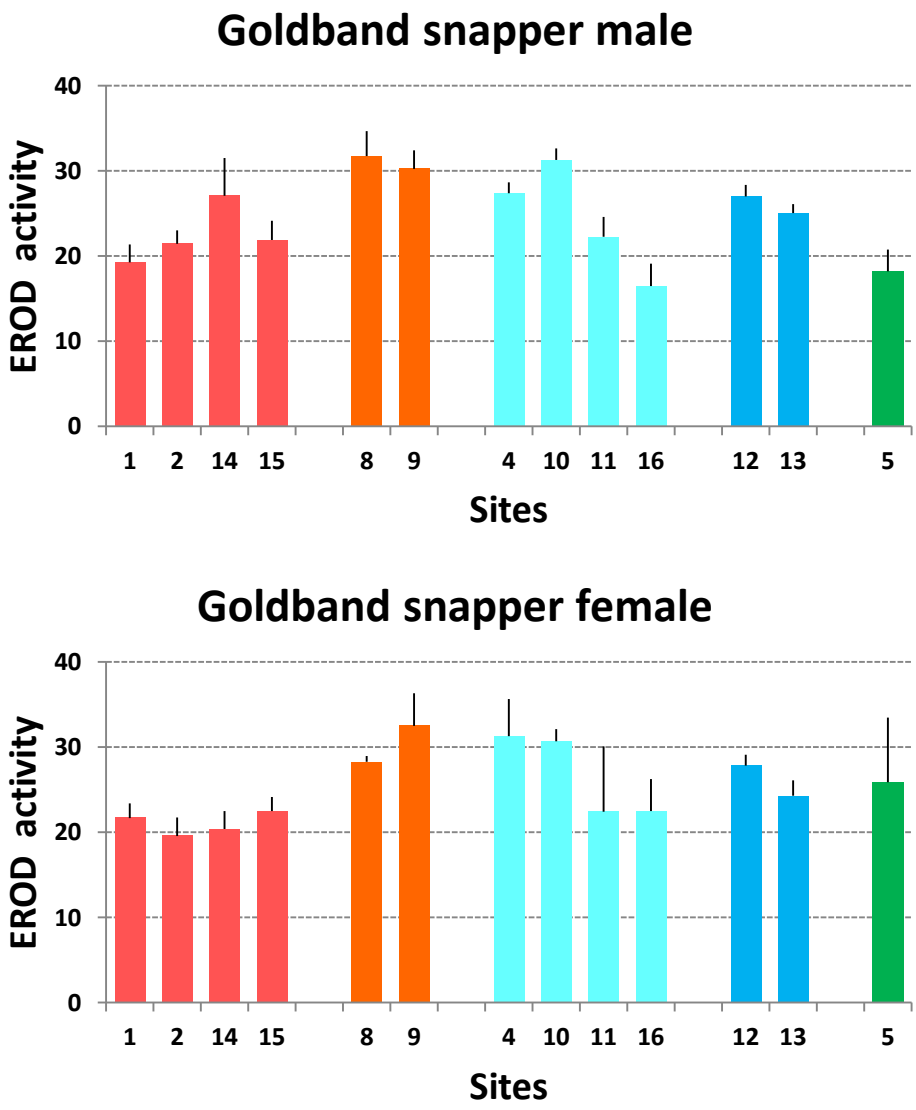



**Figure 10. EROD activity (pmol/mg protein/min) in male and female goldband snapper captured as study sites in the Timor Sea in November 2011. EROD activity in fish collected within 27 NM of the rig (sites 1, 2, 14, 15) is not statistically different (p = 0.419 and 0.700 for male and female, respectively) form EROD activity in fish from the reference Site 5. Other investigated sites were not statistically different from the reference Site 5.**

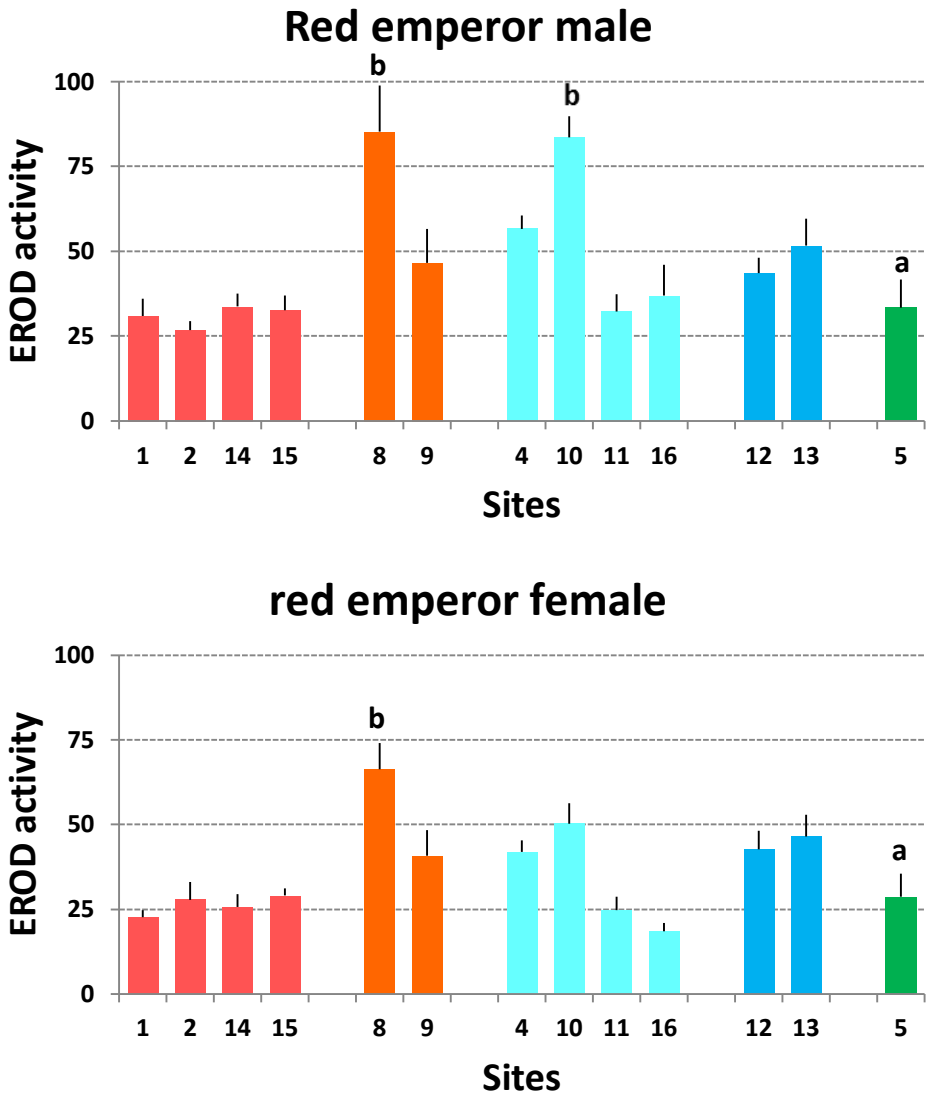



**Figure 11. EROD activity (pmol/mg protein/min) in male and female red emperor captured as study sites in the Timor Sea in November 2011. Bars with different letters indicate statistical differences. EROD activity in fish collected within 27 NM of the rig (sites 1, 2, 14, 15) is not statistically different ($p = 0.169$ and 0.605 for male and female, respectively) form EROD activity in fish from the reference Site 5. Site 8 is also significantly different form the reference Site 5 ($p < 0.001$ and $p = 0.003$ for male and female respectively).**

## Biliary Metabolites

Following oxidation of the chemical by the liver detoxification enzymes, the metabolites are directed to the biliary secretions for elimination out of the body via the intestinal route. The liquid bile secretions can therefore accumulate metabolites at levels up to 1000x more concentrated than in the surrounding environment (Hellou and Payne 1987; Meador *et al*. 1995), making this biomarker a very sensitive indicator of exposure to petroleum hydrocarbons.

As most vertebrates, fish do not accumulate petroleum hydrocarbons in their flesh because they are capable of metabolising PAHs at rates that prevent significant bioaccumulation (Hartung, 1995). Enzymes responsible for the metabolisation of PAHs do not appear to be affected by the gender of the animal and consequently, biliary metabolite levels can be combined for both male and female fish of one species. The elimination of PAH metabolites is evaluated by the measurement of three types of metabolites in the biliary secretions, these being naphthalene, pyrene and benzo(a)pyrene [B(a)P] metabolites.

In previous sampling following the Montara incident, both species of fish collected in proximity of the West Atlas well head have shown elevated PAH biliary metabolite levels relative to reference fish, in at least one occasion (Gagnon and Rawson, 2011). In Phase IV of the study however, goldband snapper and red emperor collected at sites within 27 NM from the well head no longer exhibited elevated PAH biliary metabolite levels relative to reference fish (Figure 12, Figure 13). This result, along with the low liver detoxification activity, further supports the postulate that fish are no longer exposed to petroleum hydrocarbons from the damaged underwater infrastructures.

At site 16, goldband snapper consistently showed elevated pyrene and BaP biliary metabolites. This site is located along one of the Heywood Shoal ridges, and is in close proximity to the known natural Cornea seep (Brunskill et al., 2011). The presence of this seep might be related to the ongoing positive biomarker responses observed in fish collected at Heywood Shoal.

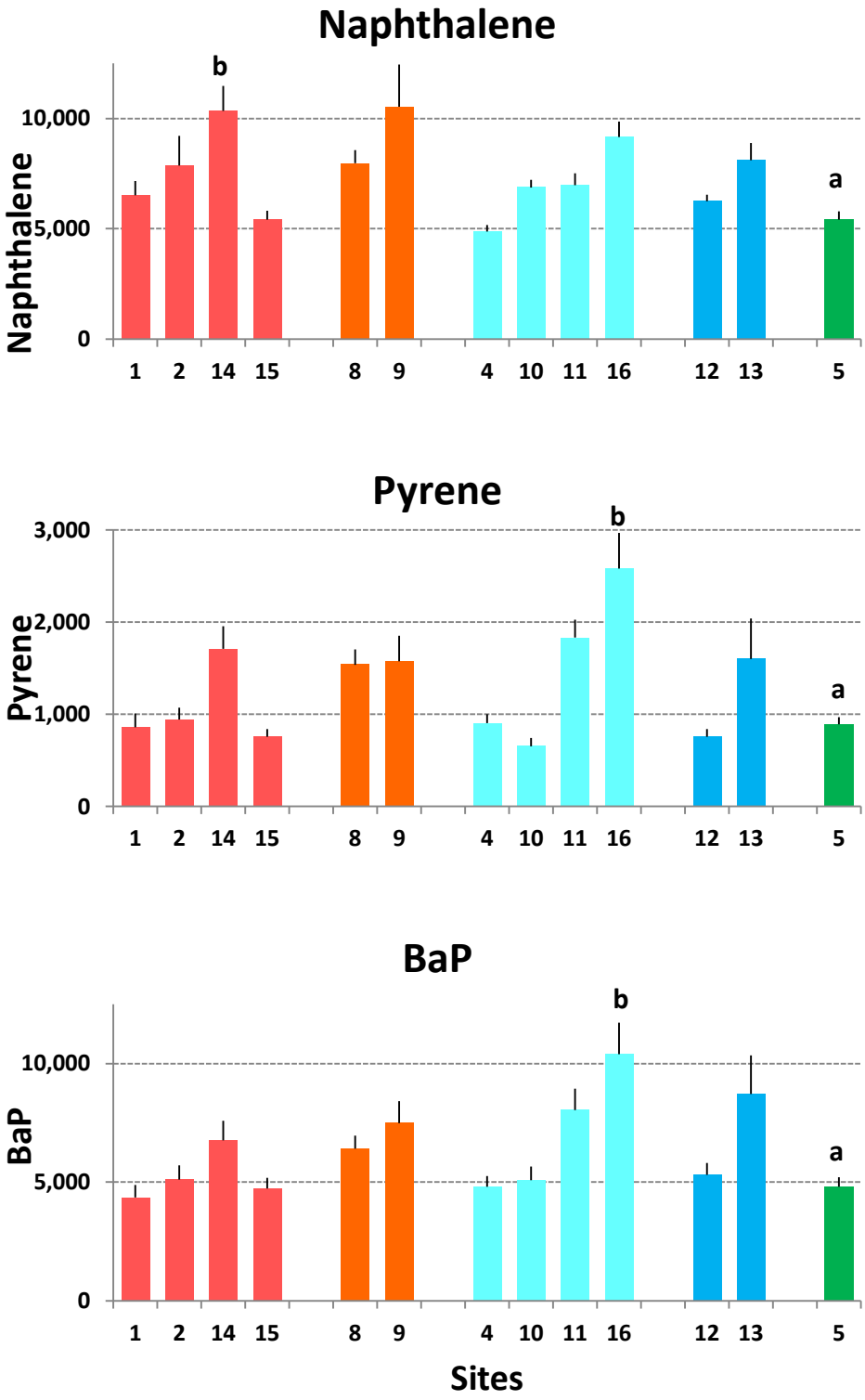



**Figure 12. Levels of naphthalene- (mg/mg protein), pyrene- (µg/mg protein) and BaP (µg/mg protein) -type biliary metabolites in goldband snapper captured at study sites in the Timor Sea in November 2011. Bars with different letters indicate statistical differences. Goldband snapper collected from study sites located within 27 NM from the well head had similar biliary metabolite levels (p =0.056, 0.062 and 0.454 for naphthalene, pyrene and BaP-type metabolites respectively) relative to fish originating from reference Site 5.**

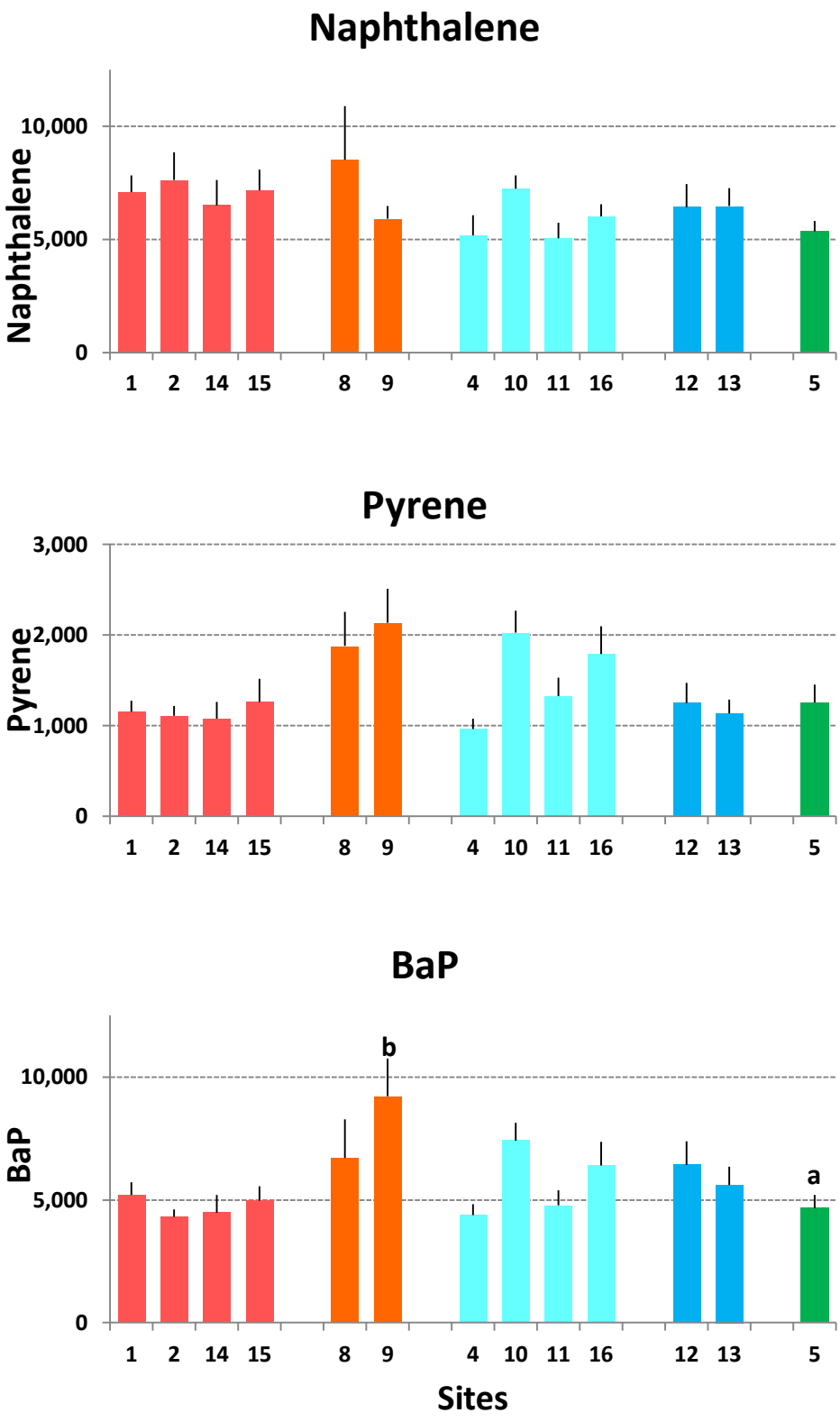



**Figure 13. Levels of naphthalene- (mg/mg protein), pyrene- (µg/mg protein) and BaP (µg/mg protein) -type biliary metabolites in red emperor captured at study sites in the Timor Sea in November 2011. Red emperor collected from study sites within 27 NM from the well head had similar biliary metabolite levels (p =0.166, 0.522 and 0.872 for naphthalene, pyrene and BaP-type metabolites respectively) relative to the fish collected at the reference Site 5.**

## Sorbitol Dehydrogenase (SDH) Activity

The enzyme sorbitol dehydrogenase is primarily found in the liver and catalyses the reversible oxidation-reduction reaction between fructose and sorbitol. Its involvement in energy metabolism is biologically relevant for metabolic purposes. In addition, the presence of SDH in the bloodstream is informative of liver damage which might follow exposure to contamination (Heath 1995). Previous work has shown that such increases in SDH in the bloodstream can follow exposure to organic contaminants including petrogenic compounds (e.g., PAHs) (Ozetric and Krajnovic-Ozetric, 1993). SDH activity is not affected by conditions such as sex of the animals or reproductive status which often are confounding factors in the interpretation of other biomarkers.

In previous investigations on the effects of the Montara incident on fish health, levels of SDH activity in the bloodstream of goldband snapper was at similar levels in exposed snapper as it was in reference fish of this species. However, immediately following the Montara hydrocarbon release, red emperor collected in impacted areas during in Phase I (November 2009) showed a significant increase in SDH activity in the blood serum, suggestive of hepatocellular damage related to exposure to petroleum hydrocarbons. The elevated SDH levels in red emperor were not observed in subsequent sampling events, indicating that liver functions in fish collected in impacted areas have returned to reference levels within 4 months post-control of the well release.

In Phase IV of the study (November 2011), sorbitol dehydrogenase activity in goldband snapper and red emperor remained comparable in fish collected from sites close form the West Atlas location and in fish collected from the reference area Figure 14). This result is in agreement with previous samplings performed at these sites, and indicates that the Montara incident has not resulted in chronic hepatic damage in fish collected within 27 NM from where the hydrocarbon release occurred.

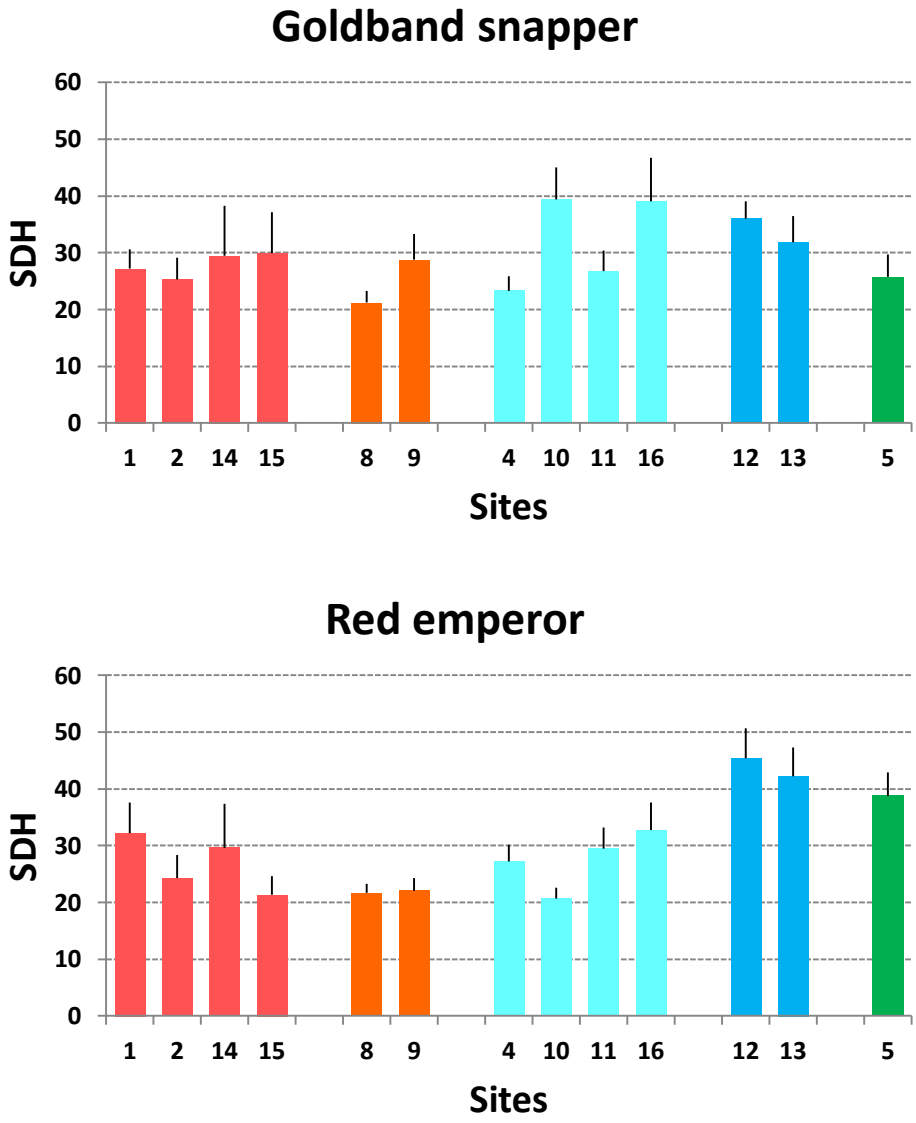



**Figure 14. SDH activity (mIU) in goldband snapper and red emperor collected from study sites in the Timor Sea in November 2011. Both species of fish collected in the vicinity of the rig (sites 1, 2, 14 15) had comparable SDH activity levels to the fish collected at the reference Site 5 (p = 0.330 and 0.101 for goldband snapper and red emperor respectively).**

## DNA damage

As noted in previous phases of Study S4A the levels of oxidative DNA damage was between 2 and 4 times higher in goldband snapper than red emperor across all sites. This is an indication of a physiological difference in either the rate of damage and endogenous repair that occurs in these fish. It is not a difference in their susceptibility to increased oxidative damage.

There were no differences between the levels of oxidative damage in goldband snapper between the study sites (Figure 15). Statistically, there were overall differences between the oxidative DNA damage in red emperor at the study sites. However, there were no trends in these differences which were associated with the location of the Montara well head. The mean oxidative DNA damage in red emperor at the reference location was close to the overall mean with no detectable differences between this and any other study site. Importantly, none of the sites closest to the Montara well head, had oxidative DNA damage levels which were above those measured in fish from the reference location (Site 5).

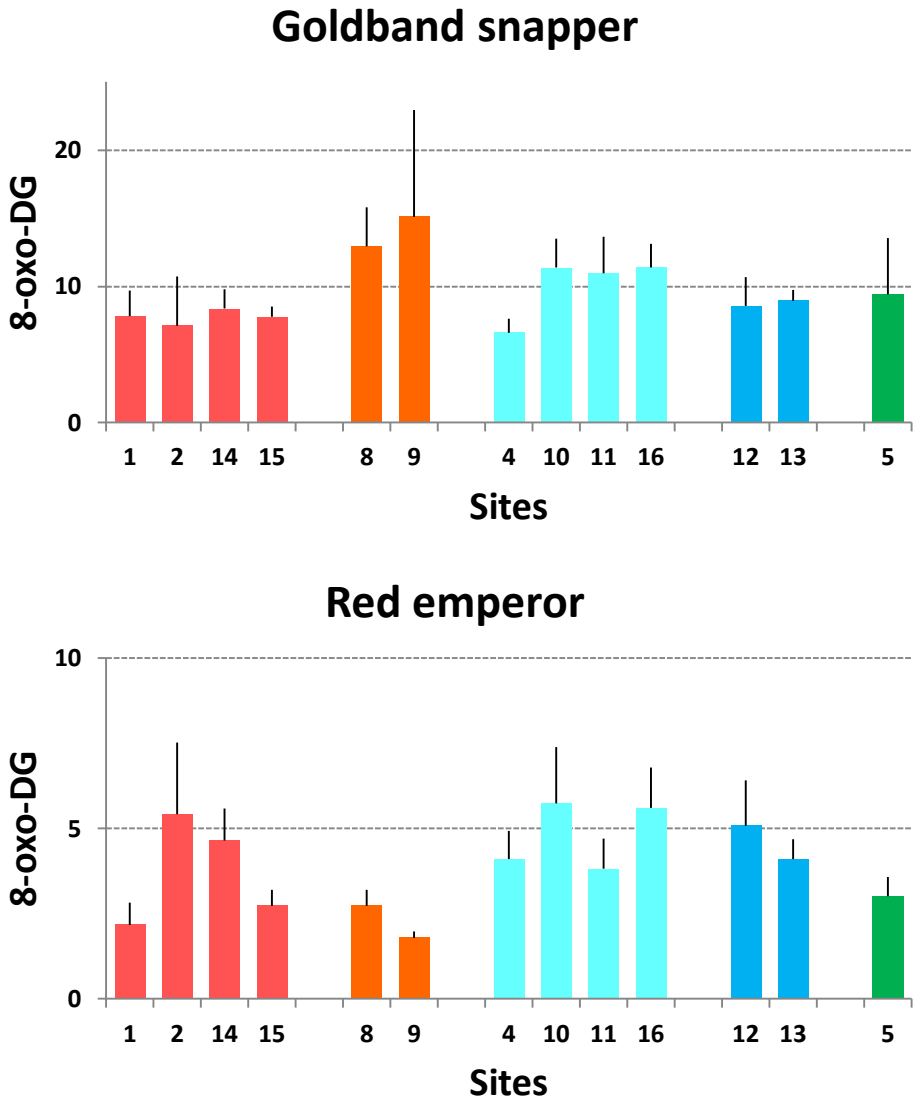



**Figure 15. Oxidative DNA damage (ng/ml/mg protein) in goldband snapper and red emperor at study sites in the Timor Sea in November 2011. No statistical differences were identified between goldband snapper collected at the study sites (p = 0.662). Between site differences were detected between red emperor collected between sites (p = 0.036) but no differences were detected between the reference site (Site 5) and any of the other study sites.**

# Conclusions

The Montara drill rig located in the Timor Sea suffered a well head accident in August 2009, resulting in the uncontrolled discharge of oil and gas condensate. The discharge commenced on 21st August 2009 and was stopped on 3 November 2009. An estimated 23,000 barrels of oil and gas condensate was released to the marine environment, coinciding with the onset of the reproductive season for the commercially important goldband snapper and red emperor.

To assess the long term potential effects of hydrocarbon exposure to fish, investigations commenced immediately after the control of the hydrocarbon release and continued for two years thereafter. To date, four sampling events have been conducted, collecting a total of 1662 fish.

The first three phases of the study found that immediately following the incident, fish collected in close proximity to the well head exhibited signs of exposure and effects however a temporal decline of effects was observed during the 12 months post-spill. While increased levels of oxidative DNA damage and increased liver size were still observed at some sites, the magnitude of the effects relative to reference areas was reduced, suggesting an ongoing trend towards normal, reference conditions.

This report describes the results of Phase IV of the monitoring program (November 2011). Two years following the end of the spill, biomarker levels in goldband snapper and red emperor had mostly returned to reference levels with the exception of the

liver somatic index (i.e. liver size relative to body size) which remained elevated in both species collected within 27 NM of the well head, relative to reference fish.

Other biomarkers of exposure such as liver detoxification enzymes and PAH biliary metabolites do not indicate recent exposure of these fish to petroleum hydrocarbons, which could have been related to this increased liver somatic index. It is possible that this long-term biomarker of exposure to contaminants has not yet returned to background conditions, or alternatively an increased liver size relative to body size could be related to environmental conditions prevailing at these sites.

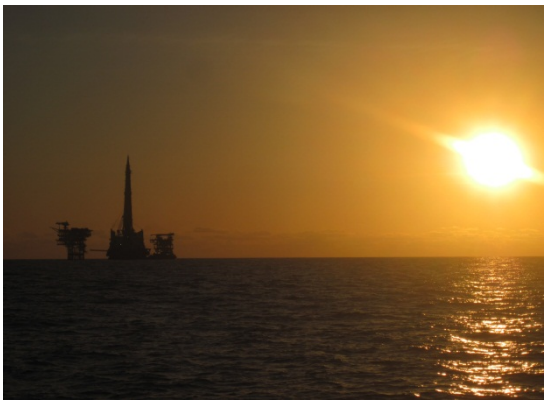
Phase IV of the monitoring program included a number of first-time sites located 30 to 80 NM form the well head. The aim of fish collection at these sites was two-fold. Firstly, sites 12, 13, 14 and 15 were sampled to provide a baseline for evaluating exposure and possible impacts on fish health, if any, once petroleum production and discharge of produced formation waters has commenced at the offshore platform. In addition to baseline biomarker levels, the sampling of these sites provided important information on the natural variability of physiological measurements and biomarker measurements that can be expected in the absence of contamination.

Secondly, newly selected sites 10, 11 and 16 were all in all in close proximity to the previously sampled Heywood Shoal (Site 4), and were added to further investigate positive biomarker responses measured in fish collected at this site during previous phases of the monitoring program. The possibility that fish collected at Heywood Shoal form a single fish assemblage is recognised, as the four sampling locations are geographically close and bathymetrically connected. Nevertheless, the four sites were discretely sampled. It was found that male red emperor collected from Site 10 showed elevated liver detoxification relative to fish collected close to the Montara well head, as well as relative to reference fish. At site 16, goldband snapper had elevated PAH biliary metabolites, indicating that fish were exposed to, and had assimilated and metabolised petroleum hydrocarbons. It is known that the Cornea seep exists in close proximity to site 16 (Brunskill et al., 2011).

Overall, measurements of physiological parameters and biomarkers of fish health in goldband snapper and red emperor two years following the Montara incident indicate that only one physiological measurement, liver-somatic index, remain elevated in fish collected within 27 NM of the drill rig. However, all other physiological parameters as well as biomarkers of fish health measured in these two species had returned to

reference levels. Phase IV of the monitoring program provided baseline data and informed on natural variability of biological parameters for future monitoring once production and PFW discharge have started at the new offshore facility.

# Recommendations



The fish health monitoring program conducted since 2009 has established that immediately following the Montara well release, the commercially important goldband snapper and red emperor collected within 80 NM of the well head were exposed to, and assimilated petroleum hydrocarbons. Fish collected within 20 NM from the well head in past surveys, had an increased liver size and occasionally, increased oxidative DNA damage. The present survey conducted two years following the incident shows that most physiological and biochemical markers measured in fish collected close to the well head have returned to reference levels. However, liver size relative to body size was significantly larger in fish collected close to the well head.

The deployment of the new processing facility to the Montara field will generate produced formation water (PFW) discharges which contain dissolved petroleum hydrocarbons as well as other industrial compounds. PAHs originating from PFW discharge can be found in fish collected in the proximity of petroleum production facilities (Gagnon, 2011). Therefore, the following recommendations are made:

**Recommendation 1:** The monitoring of fish health in the vicinity of the processing facility utilising biomarkers of exposure and effects in goldband snapper and red emperor should continue. This will provide critical information on the impact/no impact of PFW discharge on the local environment.

**Recommendation 2:** Additional samples of water and sediments should be collected in the vicinity of the processing facility to assess if PFW discharge contributes significantly to the environmental load of PAHs.

**Recommendation 3:** Yearly monitoring should be conducted until all of the biomarkers of fish health have stabilised. Subsequent monitoring should be performed biennially.

**Recommendation 4:** If significant process changes affecting the qualitative or quantitative characteristics of discharged PFW occurs, annual monitoring should be re-established.

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# Appendix I – Comments and Authors' Responses

| Comment | | Response | See Pg |
|---|---|---|---|
| **1** | Why were gonad samples not collected at Site 16? | Explanatory text added: "No samples were collected at site 16 due to several additional sites, including site 16, being sampled during the trip, and all extra preservation vials and solutions were consumed." | 24 |
| **2** | Re: suggestions of future monitoring of liver lipid content in subsequent samplings; Refer to future monitoring in later section of the report | A "Recommendations" section has been added to the report. Recommendations 3 and 4 relate to future monitoring. | 60 |
| **3** | Re: suggestions of future monitoring of the EROD biomarker in goldband snapper and red emperor. | As for Comment 2 above. | 60 |