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Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus maximus*L

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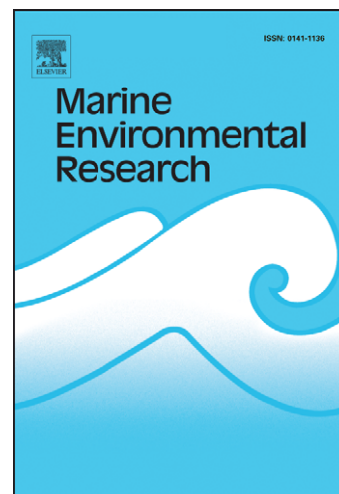
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1 Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus*
2 *maximus* L.

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4 Running title: Ecotoxicology of contaminated estuarine sediment

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19

1 Abstract

2 Hatchery-reared turbot (*Scophthalmus maximus* L.) were exposed for three weeks,
3 under laboratory conditions, to sediment collected from polluted sites in Cork
4 Harbour and a reference site at Ballymacoda, Co. Cork, Ireland. The potential of
5 surficial sediment for inducing hepatic biomarkers was assessed at two levels of
6 biological organisation: expression of cytochrome P450 [Western blotting analysis
7 and 7-Ethoxy-Resorufin O-Dealkylase (EROD), 7-Benzoxo Resorufin O-Dealkylase
8 (BROD), 7-Methoxy Resorufin O-Dealkylase (MROD), 7-Pentoxo-Resorufin O-
9 Dealkylase (PROD) activities] and DNA integrity (Comet assay). Positive controls
10 were generated, either by exposing turbot to cadmium chloride spiked seawater
11 (Comet assay) or to β -naphthaflavone by intra-peritoneal injection (cytochrome P450
12 induction).

13 The induction of cytochrome P450 activity (EROD, MROD and PROD) in animals
14 following a 7-day exposure to contaminated sediments was significantly higher than
15 those exposed to reference site sediment and remained elevated thereafter; BROD was
16 not induced. DNA single-strand breaks were also significantly higher following
17 exposure to contaminated sediments throughout the experiment. Although no direct
18 correlation between induction of alkoxyresorufin O-dealkylase activities and a
19 particular chemical class was established, the induction of MROD and PROD
20 activities in fish exposed to sediments containing complex contaminant mixtures,
21 appeared to be more sensitive than conventional EROD activity assays.

22 We conclude from the present laboratory study that *S. maximus* is a suitable sentinel
23 species for the assessment of moderately contaminated sediments and therefore allows
24 for the further development of this model for future, ecologically relevant, field
25 studies.

- 1 **Key words:** sediment; cytochrome P450; Western blotting; Comet assay;
- 2 Pleuronectiformes

ACCEPTED MANUSCRIPT

1 **1. Introduction**

2
3 Post-metamorphic Pleuronectiformes, such as turbot, maintain a close association
4 with sediments for food and cover and are therefore more likely to be exposed to
5 sediment-associated organic pollutants, including polychlorinated biphenyls (PCBs)
6 (Courtney & Langston, 1980), organotins (Hartl *et al.*, 2001) and polycyclic aromatic
7 hydrocarbons (PAHs) (Besselink *et al.*, 1998). Cytochrome P450-dependent
8 monooxygenases play an important role in the synthesis and degradation of many
9 biological molecules and the transformation of potentially carcinogenic foreign
10 compounds (Stegeman, 1989).

11 Laboratory studies with collected sediment and harbour sludge have revealed
12 induction of cytochrome P4501A1 in various benthic fish species, such as European
13 flounder (Eggens *et al.*, 1996; Besselink *et al.*, 1998) greenback flounder (Mondon *et*
14 *al.*, 2001) and plaice (Eggens *et al.*, 1996). In addition *in situ* caging experiments with
15 European flounder (Lindstrom-Seppa *et al.*, 1992; Eggens *et al.*, 1995; Beyer *et al.*,
16 1996; Eggens *et al.*, 1996; Besselink *et al.*, 1998), plaice (Eggens *et al.*, 1995; Eggens
17 *et al.*, 1996), cod (Husoy *et al.*, 1996) and dab (Sleiderink *et al.*, 1995) have
18 demonstrated that cytochrome P4501A1 induction is an environmentally relevant
19 biomarker.

20 PAHs have been shown to bind to DNA in fish, causing DNA instability and
21 potentially pre-mutagenic damage (Aas *et al.*, 2001; Myers *et al.*, 2003). However,
22 PAHs require enzymatic bioactivation in order to produce potentially damaging DNA
23 adducts (Stegeman, 1981). The alkaline single-cell gel electrophoresis assay (Comet
24 assay) can determine such pre-mutagenic DNA damage in the form of single-strand
25 breaks (Singh *et al.*, 1988). Accordingly, the Comet assay has been applied to a range
26 of fish species using aqueous exposure regimes, both *in vitro* (Devaux *et al.*, 1997;

1 Mitchelmore & Chipman, 1998a; Frenzilli *et al.*, 1999) and *in vivo* (Belpaeme *et al.*,
2 1996; Belpaeme *et al.*, 1998). Kammann *et al.* (2000) used the Comet assay to
3 examine the genotoxicity of sediment extracts to *Cyprinus carpio* leukocytes *in vitro*
4 and Nacci *et al.* (1996) determined DNA damage in flounders exposed to sediment
5 spiked with benzo(a)pyrene. In turbot, a wide variety of substrates have been found to
6 induce cytochrome P450-containing monooxygenases activity and cause DNA
7 damage, whereby the route of exposure has either been intraperitoneal injection
8 (Peters & Livingstone, 1995) or aqueous suspension (Peters *et al.*, 1997; Camus *et al.*,
9 1998).

10 Following metamorphosis, turbot display a predominantly benthic life style and
11 maintain intimate contact with sediments, where they seek shelter, waylay their prey
12 (Aarnio *et al.*, 1996; Beyst *et al.*, 1999), and are therefore likely to be exposed to
13 sediment-associated contaminants. This is of particular interest, because although
14 similar data from field experiments using other pleuronectiforme species exist (Rice
15 *et al.*, 1994; Eggens *et al.*, 1995; Myers *et al.*, 2003), there is no comparable data
16 available for turbot, *S. maximus*. Hatchery production of turbot has lead to an
17 increased availability of individuals with a known exposure history, which is an
18 essential element in any toxicological study (Boisson *et al.*, 1998).

19 The purpose of this study was to assess the effect of sediment exposure in hatchery-
20 reared *S. maximus* under controlled laboratory conditions by means of two
21 complimentary biomarkers on two levels of biological organisation, biochemical
22 (cytochrome P450 induction) and genetic (DNA instability), and to evaluate the
23 potential of this species as a model organism for the detection of sediment-associated
24 pollutants in the environment.

1 **2. Material & Methods**

2 *2.1. Site characteristics*

3 For this study, two contaminated sites Whitegate and Aghada, in Cork Harbour and a
4 reference site, Ballymacoda Estuary, outside of Cork Harbour, were chosen (Fig. 1).
5 Previous investigations, including our own, have shown that, although sediments from
6 Ballymacoda Estuary do contain metals, they are comparatively free of organic
7 contaminants, whereas those from Cork Harbour are more heavily contaminated with
8 trace metals and PAHs (Boelens *et al.*, 1999; Byrne & O'Halloran, 1999; Kilemade *et*
9 *al.*, 2004a) Table 1 summarises the chemical analysis of the sediment sampled for the
10 present study.

11 *2.2. Collection of sediment*

12 Approximately 100kg of surface sediment (the top oxygenated 0.5-1 cm) were
13 collected in June 2002, using a plastic trowel at low tide, mixed thoroughly and
14 transported to the laboratory, where sub-samples were immediately frozen for
15 chemical analysis.

16 *2.3. Sediment analyses and characterisation*

17 The <63 μ m fraction of the sediment sub-samples were analysed by ERGO
18 Umweltinstitut GmbH, Dresden, Germany (DIN EN 45001 and DIN EN ISO 9002-
19 accredited) for PAH analysis (Soxhlet extraction, GC-MS detection) and by RIVO,
20 Netherlands Institute for Fisheries Research, The Netherlands (EN/ISO 17025,
21 STERLAB accredited) for brominated flame retardants (BFR), organotin (Soxhlet
22 extraction, GC-MS detection), PCB and organochlorine pesticide (OCP) (Soxhlet
23 extraction, GC-ECD detection). The heavy metals copper, lead, cadmium and zinc
24 were analysed by atomic absorption spectrometry, following acid digestion by
25 Mercury Analytical Ltd., Limerick, Ireland, who conform to ISO 9002.

1 2.4. *Fish husbandry*

2 Juvenile turbot (0+; average weight 15.4 ± 2.77 g) were obtained from a hatchery
3 (Turbard Iarthar Chonamara Teo) in County Galway, Ireland. The fish were
4 acclimatised to aerated seawater (16°C; salinity 35; pH 8; 74 % O₂; NO₃⁻: <4 mg l⁻¹;
5 NO₂⁻: 0.25 mg l⁻¹) in 500 litre polyethylene fish-farming tanks (stocking density: 100
6 per tank) at the Aquaculture & Fisheries Development Centre in Cork for at least two
7 weeks. The water was changed every three days and the fish were fed *ad libitum* on
8 commercially available turbot pellets during the acclimation phase, but starved during
9 the experiment.

10 2.5. *Experimental design*

11 The sediment from each site was divided evenly between two 500 l seawater-seasoned
12 fish farming tanks (2 tanks for each site, 6 tanks in total) to form a layer covering the
13 floor approximately 10 cm thick. The tanks were then filled with seawater and the
14 sediment was left to settle overnight. 30 acclimated turbot were added to each tank.
15 Four turbot were sampled from the stock population at t₀ (without exposure to the
16 sediment) and then a further 4 per tank in 7-day intervals (t_{7-days}, t_{14-days}, t_{21-days}) for
17 three weeks thereafter. The fish were sacrificed by an overdose of CO₂, followed by
18 the destruction of the brain. Half the fish (two) from each tank were used for the P450
19 analysis and the other half for the Comet assay. All handling during tissue dissection,
20 dissociation, and preparations were performed on ice.

21 2.6. *Cytochrome P450 analysis – sample preparation*

22 The livers were removed, weighed, immediately shock frozen in liquid nitrogen and
23 stored at -80°C for later analysis. Individual livers were thawed on ice, homogenised
24 in ice-cold Trizma buffer (50mM, pH 7.7) and the resulting homogenate centrifuged
25 at 12,500xg (4°C). Total protein was measured according to Bradford (1976) .

1 2.6.1. *Cytochrome P450 analysis - protein detection*

2 A Western blot analysis was performed to detect expression of the cytochrome
3 P4501A1 protein. Samples were diluted in water and mixed with an equal volume of
4 sample buffer [8% Sodium dodecyl sulphate (SDS) (w/v), 20% (v/v) glycerol, 0.002%
5 (w/v) bromophenol blue, 0.125 M Tris (pH 6.8) and dithiothreitol (6 mg ml⁻¹)] to give
6 a final protein concentration of 1.3 mg ml⁻¹ and boiled for three minutes. SDS-
7 PolyAcrylamide Gel Electrophoresis (PAGE) was carried out using a mini vertical
8 electrophoresis unit (Sigma) with a 5% stacking gel/ 10% resolving gel. Samples were
9 loaded (40 µg well⁻¹) and run at 15 mA/minigel at 4°C. The resolved proteins were
10 transferred onto nitrocellulose at 4°C using a transfer buffer containing 15.7 mM Tris,
11 120 mM Glycine (pH 8.3) and 20% (v/v) methanol for 1 hour at 200V using a mini-
12 wet blotter (Sigma). The nitrocellulose membrane was incubated overnight at 4°C in
13 blocking buffer [Phosphate Buffered Saline (PBS) containing 5% (w/v) non-fat milk
14 powder] with gentle agitation. The membrane was then incubated with 1° antibody
15 diluted in blocking buffer (1:3,000 polyclonal rabbit-anti-fish P4501A1, CP-226
16 Biosense, Norway) for 3 hours at room temperature with gentle agitation. Unbound
17 antiserum was removed and the membrane was washed three times for 10 minutes
18 each in PBS containing 0.02% (w/v) Tween 20. Following this, the membrane was
19 incubated with the 2° antibody diluted in blocking buffer (1:2000 anti-rabbit IgG
20 HRP-linked antibody and 1:1000 Anti-biotin HRP-linked antibody #7075, Cell
21 Signalling) for two hours at room temperature with gentle agitation. The blots were
22 washed again as before. Bound antibodies were visualised by enhanced
23 chemiluminescent detection using Pierce SuperSignal® Substrate (Pierce, USA)
24 according to manufacturers instructions. Control samples were generated by intra-
25 peritoneal injection of either 40 mg kg⁻¹ β-naphthflavone (BNF, positive control) in

1 Dimethyl Sulfoxide (DMSO) or DMSO (a vehicle control). Livers were sampled 96
2 hours after exposure and processed as described above.

3 2.6.2. *Cytochrome P450 analysis - enzyme activities*

4 Ten μl of supernatant containing the postmitochondrial fraction were added to the
5 wells of a black fluorometric plate containing 200 μl of NADPH (0.25 mg ml^{-1}) and
6 incubated at 37°C for 10 minutes. The reaction was then started by adding 1 μl of the
7 substrates ethoxyresorufin (for EROD), benzyloxyresorufin (for BROD),
8 methoxyresorufin (for MROD) or pentoxyresorufin (for PROD) ($250 \mu\text{M}$ in DMSO)
9 and the fluorescence measured in a micro plate reader (Tecan) for 20 minutes (ex:
10 535nm; em: 590nm) according to Burke & Meyer (1974). β -Naphthaflavone, a know
11 inducer of Cytochrome P450 activity in turbot (Arukwe & Goksoyr, 1997) was used
12 as a positive control. The activity was expressed as $\text{pmol resorufin mg protein}^{-1}$
13 minute^{-1} .

14 2.7. *Comet assay*

15 Following dissection, livers for the Comet assay were prepared according to
16 (Kilemade *et al.*, 2004b). The livers were immediately washed 3 times with phosphate
17 buffered saline (PBS), gently minced with two fresh scalpel blades, transferred to
18 fresh tubes, incubated in 10 ml 0.25 % trypsin-EDTA and placed on a rotating disc at
19 room temperature for 10 minutes. The enzymatic digestion was halted by the addition
20 of an equal volume of foetal calf serum to each tube. The resultant cell suspensions
21 were subsequently decanted into fresh tubes, leaving behind the larger undigested
22 tissue pieces and centrifuged at $800 \times g$ for 10 minutes in a cooled bench-top micro-
23 centrifuge. After centrifugation, the supernatants were decanted, and the cell pellets
24 resuspended in 10 ml fetal calf serum. Following this procedure, the cell viability was
25 determined by the fluorescein diacetate/ethidium bromide (F.Da/Et.Br.) assay of

1 (Anderson *et al.*, 1994). The Comet assay was performed according to Coughlan *et al.*
2 (2002) , adapted from Woods *et al.* (1999) . Briefly, cells were sandwiched in 1%
3 low-melting agarose onto frosted microscope slides, lysed in a high salt buffer [2.5M
4 NaCl, 10mM Tris, 100mM EDTA, 1% (v/v) Triton X-100 and 10% (v/v) DMSO, pH
5 10.0, in the dark at 4°C] for 90 minutes, immersed in an alkaline solution (0.3 M
6 NaOH, 1 mM EDTA; pH 13) for 30 min at 4°C in order for the DNA to unwind and a
7 current (25V, 300 mA) applied for 25 minutes in a horizontal electrophoresis tank.
8 The pH was neutralized with Tris buffer (0.4 M Tris-HCl, pH 7.4), the DNA stained
9 with ethidium bromide and the nucleoids analysed using an epifluorescence
10 microscope (Nikon EFD-3). DNA damage was determined using the imaging analysis
11 software package Komet 4.0. (Kinetic Imaging Ltd). DNA damage was expressed as
12 percentage tail DNA (% tDNA), which is defined as the percentage DNA that has
13 migrated from the head (Hartmann *et al.*, 2003).

14 In order to substantiate the Comet assay, juvenile turbot were exposed to seawater
15 spiked with cadmium chloride (CdCl₂), an agent known to cause oxidative DNA
16 damage (Risso-de Faverney *et al.*, 2001; Valverde *et al.*, 2001). 50 l tanks of seawater
17 were spiked with 4 and 40 µM CdCl₂ respectively. A control tank consisted of
18 seawater only. Following a 40h exposure fish were sacrificed, and liver single cell
19 preparations were carried out as detailed above.

20 2.8. Data and Statistical analysis

21 Comet assay: 400-500 nuclei per treatment were analysed, i.e. 4-5 fish per duplicate
22 treatment tank – 1 slide per fish - 4-5 slides per duplicate treatment tank – 8-10 slides
23 per treatment - 50 nuclei per slide.

24 Cytochrome P450: livers from 2 fish per duplicate treatment tank.

1 The data from each group (time and site) were pooled and analysed using a non-
2 parametric Kruskal-Wallis ANOVA on ranks followed by a Dunn's pair-wise
3 multiple comparison test. A $p < 0.05$ was considered significant (Fry, 1993).

4 **3. Results**

5 *3.1. Chemical analysis of sediment*

6 The sediments used were analysed for a range of organic compounds and metals
7 (Table 1). The concentrations of some pollutants, especially total polycyclic aromatic
8 hydrocarbons (Σ PAHs) and to a lesser extent heavy metals, were substantially higher
9 in the polluted sites, Aghada and Whitegate, compared to the reference site at
10 Ballymacoda. All other compounds analysed were at or below the limit of detection at
11 all sites.

12 *3.2. Cytochrome P450 induction*

13 *3.2.1. Cytochrome P450 protein detection*

14 The Western blot analysis showed a strong immune response for cytochrome
15 P4501A1 in lanes BNF (positive control $t_{4\text{-days}}$), A (Aghada, $t_{21\text{-days}}$) and W
16 (Whitegate, $t_{21\text{-days}}$), whereas no protein recognition by the antibody was observed in
17 lanes C (control $t_{21\text{-days}}$), t_0 (pre-exposure) and B (Ballymacoda, $t_{21\text{-days}}$) (Fig. 2). This
18 agreed with the EROD activities for both positive control (BNF) and exposure
19 experiment (Figs. 3 & 4). No cross-reactivity with other proteins was observed with
20 the antibody on the Western blots.

21 *3.2.2. Cytochrome P450 activity - Positive control*

22 A significant up-regulation of EROD (cytochrome P4501A1) activity was observed in
23 turbot 96h post-intraperitoneal injection with 40 mg kg^{-1} Beta-Naphthoflavone (BNF;
24 Fig. 3).

1 3.2.3. *Cytochrome P450 enzyme activities-exposure experiments*

2 The average baseline rate of EROD, BROD, MROD and PROD activity in juvenile
3 turbot at t_0 , prior to sediment exposure, were 25.88 ± 15.6 , 0.6 ± 1.02 , 2.07 ± 0.91 and
4 1.09 ± 2.0 pmol mg protein⁻¹ min⁻¹, respectively (Fig. 4). Following 7 days of
5 exposure ($t_{7\text{-days}}$) to sediments from the reference site at Ballymacoda, the EROD
6 activity increased significantly above background levels (t_0). However, this increase
7 was substantially lower than the increase in EROD activity observed in fish exposed
8 to sediment from the contaminated sites at Aghada and Whitegate. At $t_{14\text{-days}}$ and $t_{21\text{-}}$
9 $t_{21\text{-days}}$, the EROD activity in turbot exposed to sediment from the reference site dropped,
10 but remained significantly above background levels, whilst those from both
11 contaminated sites remained elevated (Fig. 4).

12 MROD activities of fish exposed to contaminated sediments from Whitegate and
13 Aghada increased significantly above the baseline rates at t_0 (Fig. 4). With the
14 exception of $t_{21\text{-days}}$, MROD activity in fish exposed to sediment from Ballymacoda
15 did not change and were consistently significantly lower than activities of
16 contaminant-exposed fish.

17 The patterns of PROD activity for fish exposed to sediment from the reference site
18 and the contaminated sediments from Aghada, were similar to those for MROD – a
19 significant increase in PROD activity throughout the experiment for Aghada and
20 Whitegate, but no change for Ballymacoda. Although the PROD activity from
21 Whitegate was higher than Aghada at $t_{21\text{-days}}$, there was no statistically significant
22 difference between the two. BROD activity did not change throughout the
23 experiment.

1 3.6. DNA integrity

2 3.6.1. Positive control

3 A Comet assay performed on liver cells from turbot exposed for 40h to 0, 4 and 40
4 $\mu\text{mol CdCl}_2$ in aqueous suspension, showed a clear dose-related genotoxic response
5 (Fig. 5).

6 3.6.2. Comet assay

7 The cell viability following preparation of single-cell suspensions from turbot liver
8 averaged 73 %. The average background % tail DNA at t_0 , before exposure to
9 sediment, was $7.79 \% \pm 0.35$ (Fig. 6). Following 7 days of exposure ($t_{7\text{-days}}$) to
10 sediments from the polluted sites at Aghada and Whitegate, the % tail DNA values
11 increased significantly and were also significantly higher than those from the
12 reference site at Ballymacoda (Fig. 6). This pattern continued largely unchanged
13 throughout the remainder of the three-week experiment.

14 4. Discussion

15 In the present study the examination of cytochrome-P450 monooxygenase induction
16 and DNA single-strand breaks were applied, in combination, to assess the *in vivo*
17 effects of exposure to sediment-associated contaminants on the liver of juvenile
18 turbot. The availability of hatchery-reared turbot from a single cohort with known
19 exposure history allowed the determination of baseline levels for these complimentary
20 biomarkers, with the aim of developing this species as a potential model organism for
21 the contamination status of natural sediments in the field.

22 4.1. Exposure conditions

23 The predominantly benthic behaviour of turbot, together with turbulence-induced
24 agitation of the sediment surface, facilitates the re-introduction of sediment-associated
25 contaminants to the sediment-water interface and the likely exposure of wild turbot to

1 potentially harmful metals and organic compounds (Anderson *et al.*, 1987; Long *et*
2 *al.*, 1998; Schiff & Allen, 2000). Although turbot were exposed under controlled
3 laboratory conditions, experiments were designed on the basis of these observations in
4 order to simulate field conditions as closely as possible. For this reason, only the thin
5 oxygenated surface layer of sediment (1-2 cm), the layer with which turbot are most
6 likely to interact with, was collected and used in the present study.

7 4.2. Cytochrome P450 induction

8 A variety of PAHs and PCBs introduced to the aquatic environment are known to
9 induce cytochrome P450 in turbot (Peters & Livingstone, 1995; Peters *et al.*, 1997;
10 Boleas *et al.*, 1998; Aas *et al.*, 2000). Baseline cytochrome P450 activity may vary
11 considerably and depend on a variety of factors, such as age, sex, sexual maturity,
12 season, exposure history and diet. This is reflected in the baseline EROD activity of
13 juvenile turbot (15 g) measured in the present study ($25 \pm 15 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$)
14 ¹), compared with the previously reported values of $10.8 \pm 2 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$
15 for 90 day old juveniles, $12.3 \pm 4 \text{ pmol min}^{-1} \text{ mg protein}^{-1}$ for sexually mature adults
16 (Peters & Livingstone, 1995).

17 In this study the 21-day exposure of juvenile turbot to polluted sediments showed
18 EROD activities significantly above baseline values and also above those from fish
19 exposed to sediments from a reference site (Fig. 4). Significant EROD induction was
20 observed after 7 days and the activity remained elevated thereafter. Although there are
21 no comparable data available for turbot, exposure experiments of other benthic fish
22 species to contaminated whole sediments, in both field or laboratory studies, have
23 yielded similar results: *Limanda limanda*, wild catch field study (Sleiderink *et al.*,
24 1995); *Platichthys flesus*, whole sediment mesocosm study (Besselink *et al.*, 1998); *P.*
25 *flesus* and *Gadus morhua*, wild catch and hatchery-reared caged field studies (Beyer

1 *et al.*, 1996; Husoy *et al.*, 1996); *P. flesus* and *Pleuronectes platessa*, wild catch and
2 caged field studies (Eggens *et al.*, 1995; Eggens *et al.*, 1996); *Rhombosolea tapirina*,
3 hatchery-reared whole sediment laboratory study (Mondon *et al.*, 2001); *Cottus*
4 *cognatus*, wild catch whole sediment laboratory study (Tetreault *et al.*, 2003).

5 A variety of PAHs, planar PCBs, chlorinated dioxins and furans are known inducers
6 of cytochrome P4501A1 (Stegeman & Hahn, 1994).

7 In complex chemical mixtures, such as those found in the polluted sediments from
8 Aghada and Whitegate, any cytochrome P450 upregulation is likely to be the net
9 result of additive, synergistic or antagonistic chemical interactions. Although the
10 precise nature of these interactions was beyond the scope of the present study, the
11 prevalence of Σ PAHs (Table 1) in the sediments samples suggests that PAHs were
12 probably mainly responsible for the observed increase in EROD activity.

13 Although background levels of EROD activity were detected in t_0 and reference site
14 samples (Fig. 4), on the corresponding Western blot there is no band for t_0 and only a
15 very faint band for the reference site (Fig. 2). This apparent discrepancy is due to the
16 fact that protein expression in samples from Aghada and Whitegate, as well as the
17 BNF samples, was very high and the antibody dilutions and the exposure time during
18 film development were optimised accordingly. The MROD activity also showed a
19 significant increase in turbot exposed to sediment from Cork harbour (Fig. 4). In
20 contrast to mammals, knowledge of multiple cytochrome P450 enzymes in fish is
21 limited and controversial. It has been suggested that unlike mammals, where there is a
22 clear distinction between cytochrome P4501A1 and cytochrome P4501A2, there is
23 only one cytochrome P4501A enzyme in fish (Stegeman, 1989). Smeets *et al.* (2002)
24 reported MROD activity in *P. flesus*, *L. limanda*, *Oncorhynchus mykiss* and
25 *Microstomus kitt*, but concluded from strong correlations between EROD and MROD

1 activities, that these were linked, suggesting, that there was only one cytochrome
2 P4501A enzyme involved. In contrast cytochrome P4501A2-like induction has been
3 reported from a variety of fish species, such as *P. platessa* (Leaver *et al.*, 1988),
4 *Pagrus major* (Mizukami *et al.*, 1994), *P. flesus* and *Anguilla Anguilla* (Rotchell *et*
5 *al.*, 2000). The correlation between EROD and MROD activities from turbot in the
6 present study ($r = 0.81$) and reports by (Celander & Forlin, 1992) and Berndtson &
7 Chen (1994) of two cytochrome P4501A enzymes in *O. mykiss* suggest that the
8 induction of cytochrome P4501A2 in fish may indeed be species specific.

9 PROD activity was also significantly increased in turbot exposed to Cork Harbour
10 sediment (Fig. 4). PROD in mammals is associated with cytochrome P4502B. PROD
11 activity has been detected in several marine and freshwater fish species exposed to
12 water-borne pollutants, including *Cyprinus carpio* (Machala *et al.*, 1997), *O. mykiss*
13 (Lindstrom-Seppa *et al.*, 1992; Haasch *et al.*, 1994), and immunological detection of
14 P4502B has been demonstrated for *Stenotomus chrysops* (Stegeman, 1989). To our
15 knowledge this is the first time induction of PROD activity has been recorded in fish
16 exposed to sediments containing complex mixtures of contaminants. Typical PROD
17 inducers are barbiturates, non-planar PCBs and DDT. Failure by Yawetz *et al.*
18 (1998a) to detect cytochrome P4502B induction in *Mugil capito* exposed to
19 cytochrome P4501A1 inducers indicates a certain degree of substrate specificity of
20 this enzyme in fish. However, as the above-mentioned compounds were barely
21 detectable in sediments from Aghada and Whitegate (Table. 1), it is unclear what
22 caused the induction of cytochrome P4502B in the present study.

23 BROD activity shows a broader specificity and is a known marker for cytochromes
24 P4501A, P4502B and P4503A in mammals. Cytochrome P4503A is also known to
25 occur in various fish species, such as *M. capito* (Yawetz *et al.*, 1998b), *G. morhua*,

1 (Husoy *et al.*, 1996) and *Fundulus heteroclitus* (Celander & Stegeman, 1997) and can
2 be induced by cytochrome P4501A inducers (Yawetz *et al.*, 1998b). Failure to detect
3 BROD activity in the present study suggests that the induction of this enzyme is not
4 only less specific but may also be less sensitive than EROD, MROD and PROD and
5 therefore presumably less suitable as a biomarker for sediment contamination in
6 turbot.

7 Baseline values for MROD and PROD activities in fish have generally been found to
8 be much lower than those for EROD (Yawetz *et al.*, 1998a; Kennedy *et al.*, 2003) and
9 this was also observed in the present study (Fig. 4). Although the EROD activities
10 presented here are approximately three to four times higher following sediment
11 exposure than those for MROD and PROD, the relative increase in activity above
12 baseline is much higher for MROD and PROD (Table 2). This indicates that induction
13 of MROD and PROD, as well as EROD in liver tissue samples can be suitably
14 adopted to evaluate the effects of exposure of turbot to sediment-associated organic
15 contaminants. However, a direct correlation of these activities with specific isoforms
16 of cytochrome P450 in turbot remains to be established.

17 4.3. DNA integrity

18 In order to confirm that the Comet assay can detect DNA damage in turbot exposed to
19 a known genotoxic agent, a control study was first carried out exposing a sub-sample
20 of the turbot employed in the sediment-exposure to seawater spiked with CdCl₂, a
21 substance known to produce DNA damage (Pruski & Dixon, 2002). Figure 5 clearly
22 demonstrates CdCl₂-induced single-strand breaks above background levels in liver
23 cells of exposed turbot.

24 In the present study the background values in juvenile turbot averaged 4.10 % ± 0.18
25 tail DNA. These results agree well with the 4 % and 5 % background reported in

1 isolated trout hepatocytes by Mitchelmore and Chipman, (1998a, b), respectively, and
2 4 % in liver cell suspensions from juvenile turbot (Belpaeme *et al.*, 1998).

3 The increase in DNA single strand breaks in turbot was significant following a 7-day
4 exposure to contaminated sediments from both Aghada and Whitegate (Fig. 6). The
5 observation, that % tail DNA values remained elevated after 14 and 21 days and that
6 the values in fish exposed to the contaminated sediments remained consistently higher
7 than those exposed to sediments from the reference site at Ballymacoda, suggest that
8 beyond a seven day exposure, their genotoxic response was not time-related. This
9 seems to support the findings of Deventer (1996) who reported that DNA damage in
10 isolated blood cells of *Brachydanio rerio*, exposed to methyl methane sulphonate,
11 increased initially and leveled off at around 96 hours. Furthermore, Belpaeme *et al.*
12 (1998) reported significant DNA damage in turbot liver cell preparations exposed to
13 ethyl methane sulphonate occurring only after 7 days exposure.

14 As mentioned above, many organic contaminants, including PAHs, undergo
15 bioactivation by the monooxygenase system (Stegeman, 1981), producing reactive
16 intermediates, including free radicals, which are known to form DNA adducts and
17 cause oxidative DNA damage (Canova *et al.*, 1998, Nacci *et al.*, 1992; Nacci *et al.*,
18 1996; Mitchelmore *et al.*, 1998, Xue and Warshawsky, 2005.), that can lead to an
19 increase in the level of single strand breaks. In addition, organic contaminants,
20 including PAHs, may cause an induction of the cytochrome P450 enzyme activity in
21 exposed organisms (Myers *et al.*, 2003). However, an up-regulation of cytochrome
22 P450, following exposure to complex contaminant mixtures, does not necessarily
23 imply DNA damage, because some cytochrome P450 inducers, such as certain
24 dioxins, are not genotoxic. Furthermore, not all genotoxic compounds are cytochrome
25 P450 inducers (e.g. CdCl₂). Thus, although the observed effects in both assays used in

1 this study are probably mainly caused by the elevated levels of PAHs, these endpoints
2 should be regarded as complementary.

3 **5. Conclusions**

4 The level of contamination in polluted sediments used here was, by comparison with
5 other sites in Ireland and the British Isles, relatively low (Widdows *et al.*, 2002).

6 Therefore, the present laboratory study demonstrates that *S. maximus* could be used as
7 a sentinel species for the assessment of even moderately contaminated sediments and
8 therefore allows for the further development of this sensitive model for future field
9 studies.

10 Although no direct correlation between XROD induction and a particular chemical
11 class was established here, the induction of MROD and PROD activities in fish
12 exposed to sediments containing complex contaminant mixtures, were shown to be
13 more sensitive than conventional EROD activity.

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1 Table 1
2 Sediment characterisation and chemical analysis.
3

| | Ballymacoda (Site 1) | Whitegate (Site 2) | Aghada (Site 3) |
|---------------------------------|----------------------|--------------------|-----------------|
| Dry weight (%) | 57 | 49 | 46 |
| TOC (%) | 3.11 | 2.77 | 3.66 |
| < 63 μm fraction (%) | 75.15 | 57.66 | 76.66 |
| Organics (ng g^{-1}) | | | |
| Σ PAH | 528.30 | 924.40 | 1000.7 |
| Σ PCB | 2.90 | 3.20 | 3.40 |
| Σ OCP | 3.31 | 3.22 | 3.25 |
| Σ BFR | 0.50 | 0.80 | 4.70 |
| Σ Organotin | 4.90 | 9.50 | 12.50 |
| Metals ($\mu\text{g g}^{-1}$) | | | |
| Cadmium | <0.1 | <0.1 | <0.1 |
| Copper | 8.1 | 13.3 | 27 |
| Lead | 15.6 | 16.4 | 18 |
| Zinc | 78.6 | 99.1 | 105.3 |

4
5 TOC: Total Organic Carbon; PAH: Polycyclic Aromatic Hydrocarbons; PCB: Polychlorinated
6 Biphenyls; OCP: Organochlorine pesticide; BFR: Brominated Flame Retardants.

1 Table 2
 2 Increase of cytochrome P450
 3 enzyme activity above t_0 , following
 4 exposure to sediment from the
 5 reference and contaminated sites ($t =$
 6 days).
 7

| | | % increase from t_0 | | |
|------|-----------|-----------------------|----------|----------|
| | Site | t_7 | t_{14} | t_{21} |
| EROD | Reference | 135 | 65 | 59 |
| | Aghada | 444 | 335 | 393 |
| | Whitegate | 427 | 393 | 460 |
| BROD | Reference | 80 | 75 | 68 |
| | Aghada | 80 | 37 | 65 |
| | Whitegate | n.d.* | 67 | 71 |
| MROD | Reference | 41 | 22 | 905 |
| | Aghada | 1904 | 1913 | 1722 |
| | Whitegate | 1435 | 2370 | 2414 |
| PROD | Reference | 82 | 43 | 141 |
| | Aghada | 1514 | 1438 | 2489 |
| | Whitegate | 1574 | 1775 | 4210 |

8 *) no data

1 Fig. 1. Sediment sampling sites (1) Reference site at Ballymacoda; contaminated sites
2 in Cork Harbour (2) Whitegate and (3) Aghada.

3

4 Fig. 2. Western blot analysis of cytochrome P4501A1 expression in liver samples of
5 turbot following a 21 day exposure to contaminated sediments. MW, molecular
6 weight marker; BNF, β -naphthoflavone positive control; C, control; t_0 , before
7 exposure to contaminated sediments; B, Reference site at Ballymacoda; A,
8 contaminated site at Aghada and W, Whitegate.

9

10 Fig. 3. Validation of P450 activity (EROD) assay using BNF (40 mg kg⁻¹ *i.p.* in
11 DMSO) as a positive control. * indicating significant difference; n = 5; (P < 0.05).

12 Mean \pm standard deviation (SD)

13

14 Fig. 4. P450 activity before and after exposure to sediments from the Reference site a
15 Ballymacoda and the contaminated sites at Aghada and Whitegate. EROD,
16 7-ethoxyresorufin-O-deethylase; PROD, 7-pentoxoresorufin O-depentylase;
17 BROD, 7-benzyloxyresorufin O-debenzylase MROD, 7-methoxyresorufin
18 O-methoxyresorufin. ** indicating significant difference between the Reference site
19 at Ballymacoda and t_0 (P < 0.05); * indicating significantly higher values in the
20 contaminated site at Aghada than Ballymacoda and t_0 (P < 0.05); † indicating
21 significantly higher values in the contaminated site at Whitegate than Aghada,
22 Ballymacoda and t_0 (P < 0.05); n = 8. Mean \pm SD

23

24 Fig. 5. Validation of the Comet assay performed on liver single-cell suspensions using
25 CdCl₂ as a positive control. * indicating significant difference between 0 and 4 μ M (P

1 < 0.05); † indicating significantly higher values at 40 than 0 and 4 μ M (P < 0.05); n =

2 5. Mean \pm SD

3

4 Fig. 6. Comet assay showing the percentage DNA in the tail in liver single-cell
5 suspensions from turbot before and after exposure to sediments from the Reference

6 site a Ballymacoda and the contaminated sites at Aghada and Whitegate. * indicating

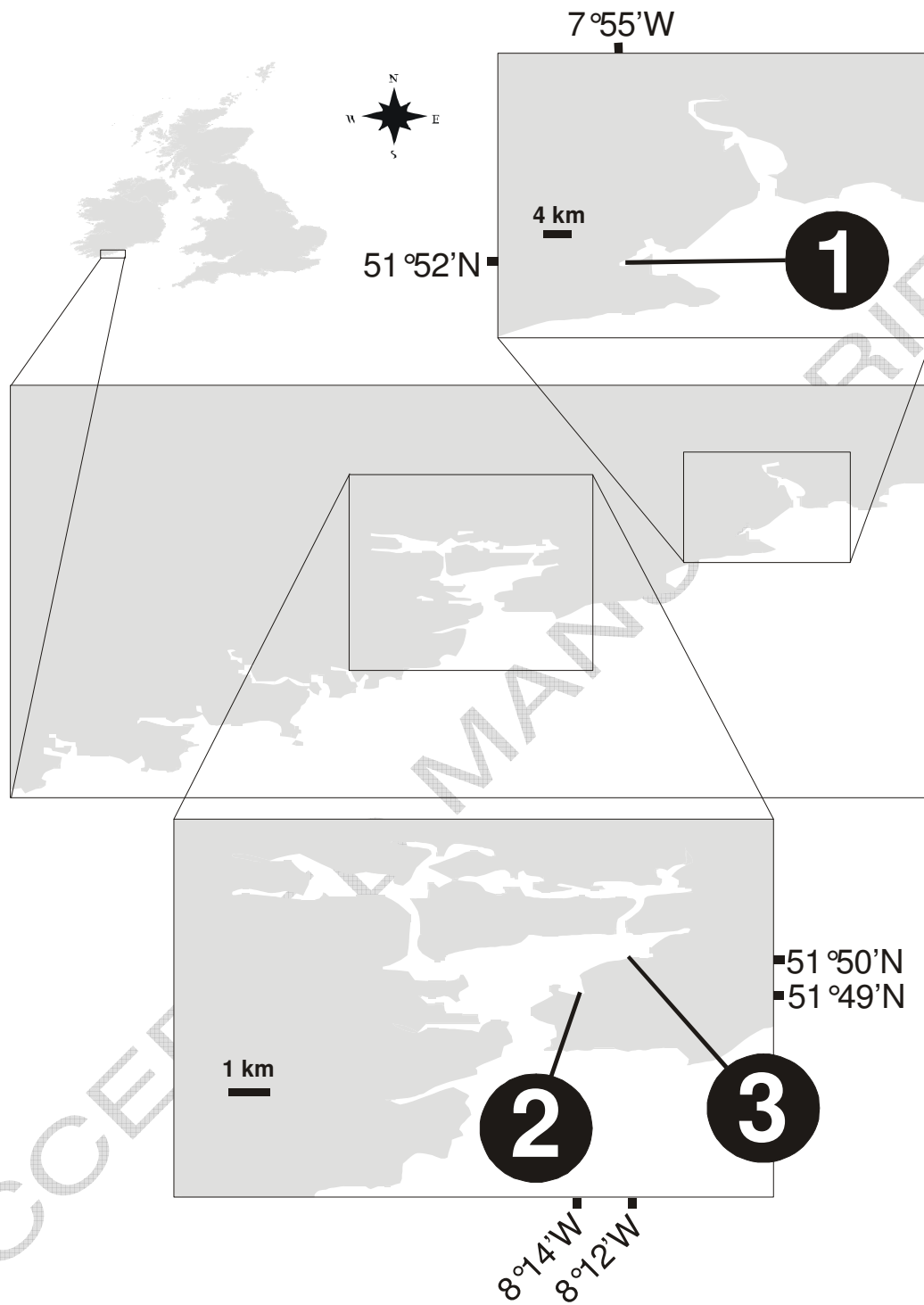
7 significant difference between the Reference site at Ballymacoda and t_0 (P < 0.05); †

8 indicating significantly higher values in the contaminated site at Aghada than

9 Ballymacoda and t_0 (P < 0.05); †† indicating significantly higher values in the
10 contaminated site at Whitegate than Aghada, Ballymacoda and t_0 (P < 0.05); n = 8.

11 Mean \pm SD

12



1
2 Fig. 1

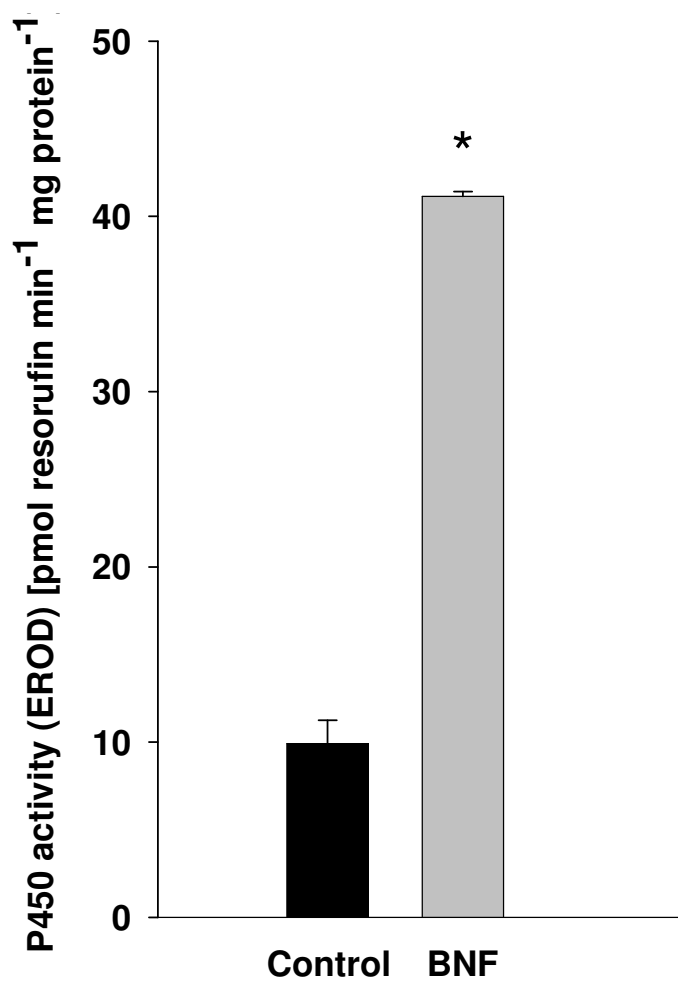
1
2
3
4
56
7
8

50 kDa →

MW BNF C t_0 B A W

Fig. 2

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1
2
34
5 Fig.3

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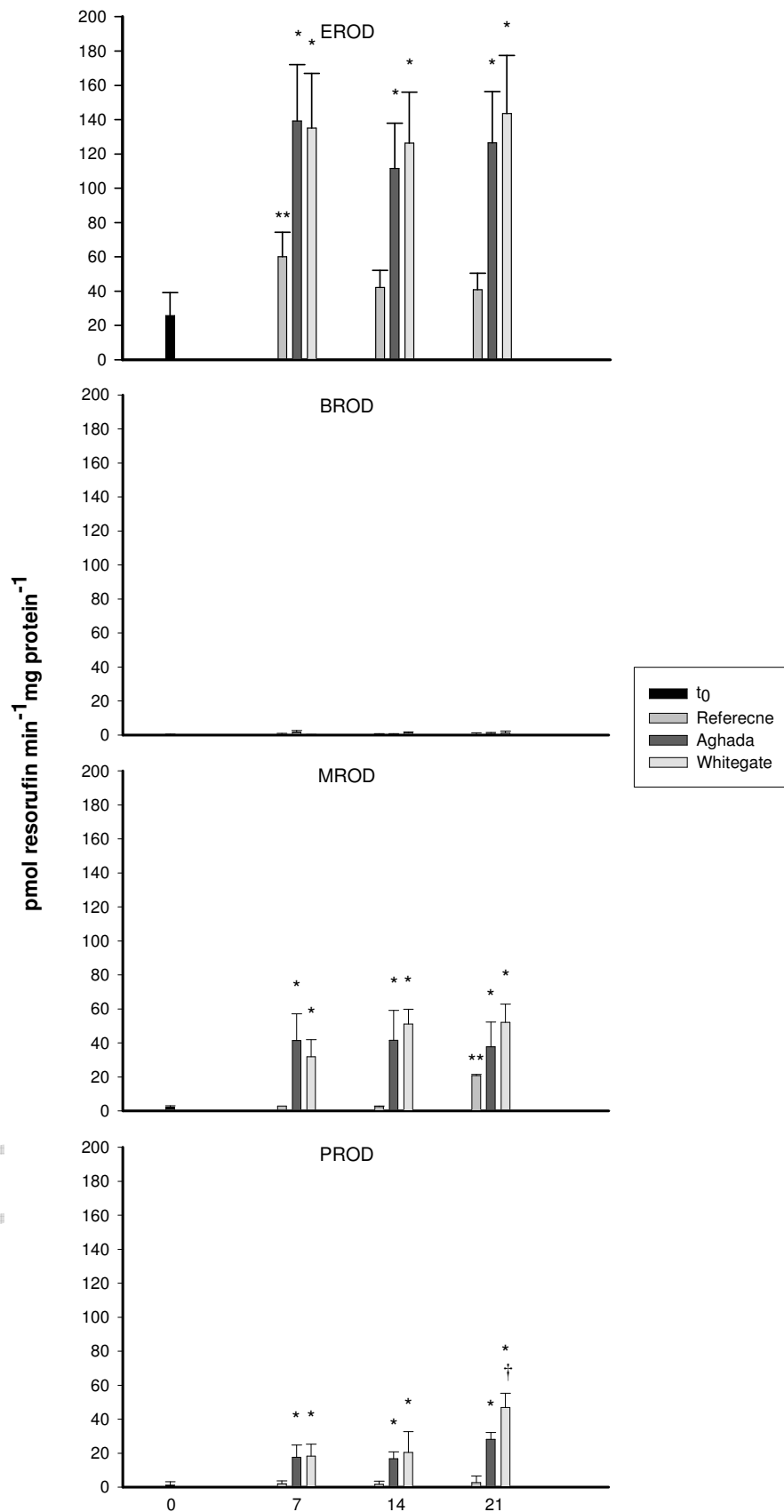
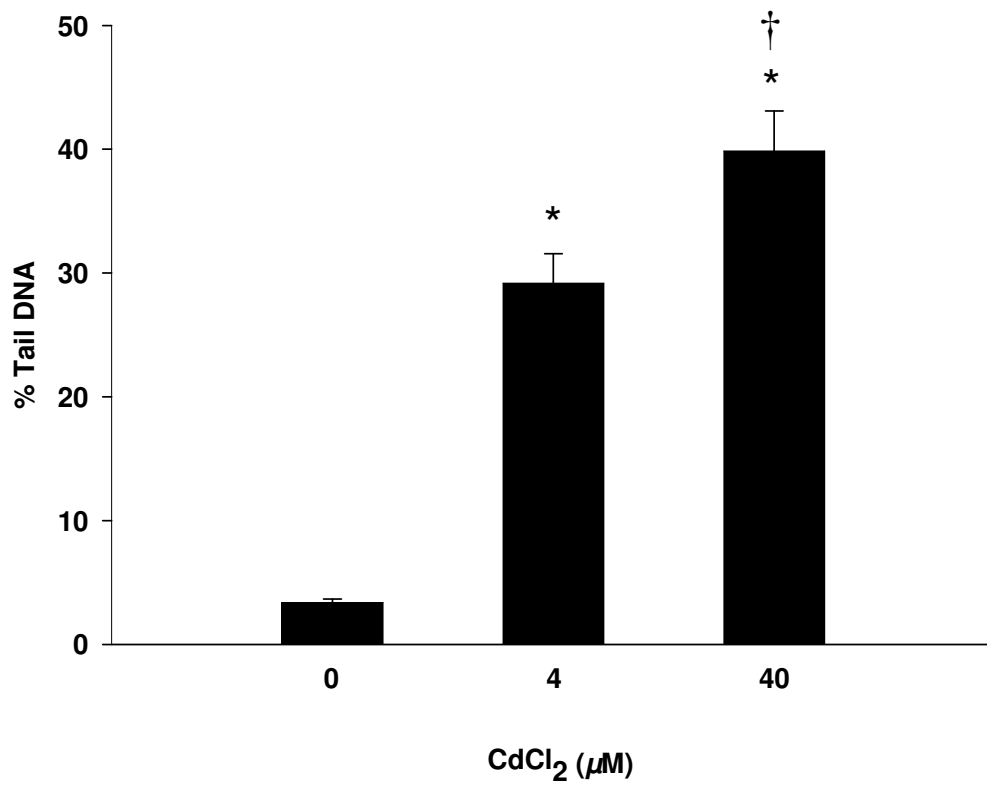
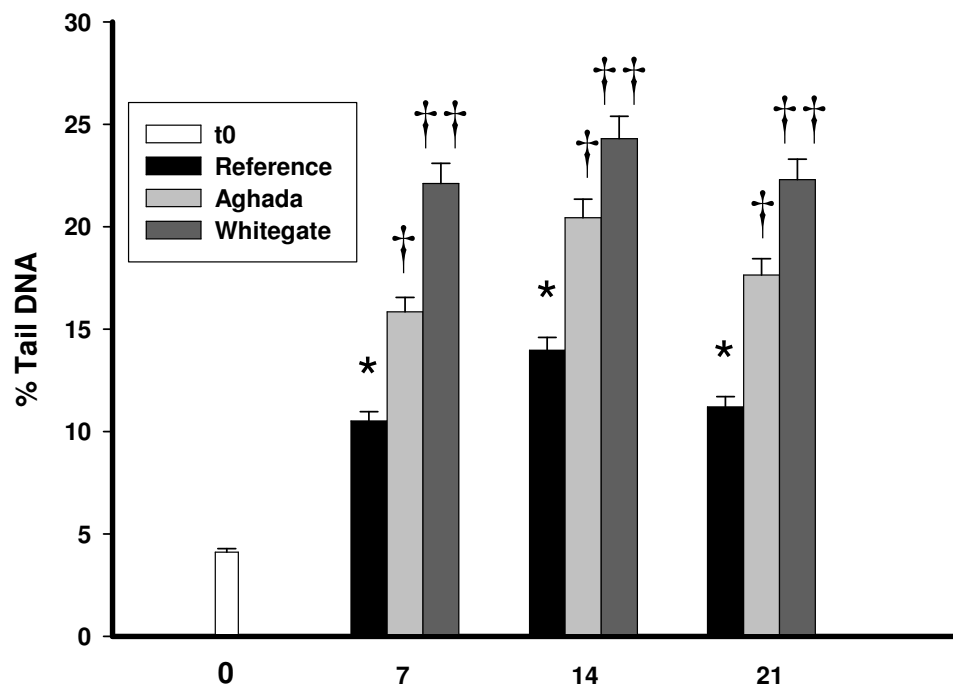


Fig. 4



1
2 Fig. 5.
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Fig.6

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