Molecular evolution and functional characterisation of tunicate xenobiotic receptors

BY

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Abstract

Marine microorganisms generate a wide range of 'bioactive' compounds that can have far-reaching effects on biological and ecological processes. Metazoans have developed specialised biochemical pathways that metabolise and eliminate potentially toxic chemicals (xenobiotics) from their bodies. The vertebrate xenobiotic receptor, pregnane X receptor (PXR), is a ligand-activated nuclear receptor transcription factor regulating expression of multiple detoxification genes. Ligand-binding domains (LBDs) of vertebrate PXR orthologues may have adaptively evolved to bind toxins typically encountered by these organisms. Marine invertebrate filter-feeders are exposed to relatively high concentrations of xenobiotics associated with their diet. Tunicates (phylum: Chordata) are of particular interest as they form the sister clade to the Vertebrata. Genomes of the solitary tunicate *Ciona intestinalis* and the colonial tunicate *Botryllus schlosseri* both encode at least two xenobiotic receptors that are orthologues to both the vertebrate vitamin D receptor (VDR) and PXR.

Pursuing the idea that tunicate xenobiotic receptors (VDR/PXR) may adaptively evolve to bind toxic chemicals commonly present in an organism's environment, this thesis aims to identify if: (i) adaptive evolution is acting on putative tunicate VDR/PXR orthologues to enhance binding of dietary xenobiotics; (ii) these receptors are activated by dietary xenobiotics (e.g. microalgal biotoxins) and; (iii) tunicate VDR/PXR LBDs can be used as sensor elements in yeast bioassays for the detection of both natural and synthetic bioactive compounds. To identify genetic variation and to search for evidence of positive selection, next-generation sequencing was performed on three tunicate VDR/PXR orthologues genes. Recombinant yeast (*Saccharomyces cerevisiae*) cell lines were developed for the functional characterisation of tunicate VDR/PXR LBDs. These tunicate VDR/PXR LBD-based yeast bioassays were utilised to detect known microalgal biotoxins, natural bioactive compounds, and environmental contaminants.

Next-generation sequencing revealed both an unusually high genetic diversity and strong purifying selection in VDR/PXR orthologues from *C. intestinalis* and

B. schlosseri. Single-base-deletion allelic variants were found in *C. intestinalis* VDR/PXR orthologues resulting in predicted proteins having a DNA-binding domain but lacking a LBD. The persistence of these variants may reflect constitutive expression of detoxification genes as a selective advantage in the marine environment.

To assess the functional characteristics of tunicate VDR/PXR orthologues, recombinant yeast cell lines were developed that express VDR/PXRα LBDs from *C. intestinalis* and *B. schlosseri*. These chimeric proteins mediate ligand-dependent expression of a *lacZ* reporter gene which encodes an easily assayed enzyme (β-galactosidase). These yeast bioassays were highly sensitive towards both synthetic and natural toxins (coefficients of variance, CV <25%). Microalgal biotoxins (okadaic acid and portimine) were two orders of magnitude more potent than synthetic chemicals, which was consistent with the hypothesis that tunicate xenobiotic receptors can bind marine bioactive compounds frequently present in a filter-feeder's diet.

Following these functional studies, the yeast bioassays were tested in a more applied context by screening the following compounds: (i) natural bioactive compounds that represent promising compounds for drug development and; (ii) synthetic chemicals that are common environmental pollutants. Of the 34 compounds tested, 30 were active in the tunicate yeast bioassays. The yeast bioassays were particularly sensitive towards a small number (n = 11) of marine and terrestrial bioactive compounds (CJ-13-014, CJ-13-104, thysanone and naringin) and emerging contaminants such as pharmaceuticals (ketoconazole), antifungals (radicicol), preservatives (butylated hydroxtoluene) and surfactants (oil dispersants), generating CV values <25%. Activities of the remaining 19 compounds were highly variable and appeared to depend on several factors, such as solvent used, duration of exposure and type of recombinant protein expressed (e.g. *C. intestinalis* versus *B. schlosseri* VDR/PXRα).

In conclusion, the yeast bioassay developed in this thesis, with further development, may provide a template for novel bioassays that may find application in routine microalgal biotoxin testing and environmental monitoring. These bioassays may also assist in the identification of marine bioactive compounds as drug lead compounds.

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List of Publications

This thesis is composed of a series of independent manuscripts. For all manuscripts, I conceived the questions/experiments in consultation with my thesis supervisor, conducted the data analyses and drafted each manuscript. My primary thesis supervisor Dr Andrew Fidler (Cawthron Institute) reviewed each draft manuscript and gave advice, and some assistance, with experimental and laboratory work. I collected all field samples and conducted the laboratory work, unless otherwise stated.

Chapter One has been published as:
Richter, I & Fidler AE (2014) Marine invertebrate xenobiotic-activated nuclear receptors: their application as sensor elements in high-throughput bioassays for marine bioactive compounds. Marine Drugs. 12(11): 5590-618. doi: 10.3390/md12115590. IF: 3.512

Chapter Two has been published as:
Richter, I & Fidler AE (2015) Transcripts of tunicate orthologues of the pregnane X receptor (PXR): characterisation and natural variation. Marine Genomics. doi: 10.1016/j.margen.2015.05.006. IF: 1.971

Chapter Three has been published as:
Richter, I & Fidler AE (2015) Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast. Toxicon 95(0): 13-22. doi: 10.1016/j.toxicon.2014.12.011. IF: 2.381

Chapter Four is being prepared as a manuscript for submission with the proposed title:
Richter, I & Fidler AE. Novel yeast bioassays based on tunicate xenobiotic receptors for high-throughput screening of natural bioactive compounds. Sensors. IF: 2.048

Chapter Five represents a possible future publication.

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Dr Ayelet Voskoboynik (Stanford University, U.S.A.) kindly provided access to unpublished *B. schlosseri* genomic sequence data. Prof Baruch Rinkevich (National Institute of Oceanography) shared his knowledge about *B. schlosseri*. Rod Asher (Cawthron Institute) provided assistance with dissections and gave advice on *Ciona* morphology (Chapter Two). Andy Selwood and Dr Lincoln MacKenzie (both Cawthron Institute) provided microalgal toxin standards (Chapter Three). Amanda Heapy and Prof Margaret Brimble (both University of Auckland, New Zealand) provided natural bioactive chemicals (Chapter Four). I would like to thank Alexis Simon (École Normale Supérieure, France) for laboratory assistance (Chapter Five).

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“A lot of people have gone further than they thought they could because someone else thought they could.”

Richard Branson

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List of Abbreviations

AD	Activation domain
ADH1	alcohol dehydrogenase 1
AF-2	Activation function domain 2
AhR	Aryl hydrocarbon receptor
ANOVA	One-way analysis of variance
Akt	Protein kinase B
BaP	Benzo[α]pyrene
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BPA	Bisphenol-A
*Bs*VDR/PXRα	*Botryllus schlosseri* VDR/PXR orthologue α
CAR	Constitutive androstane receptor
95% CI	95% confidence interval
*Ci*VDR/PXRα	*Ciona intestinalis* VDR/PXR orthologue α
*Ci*VDR/PXRβ	*Ciona intestinalis* VDR/PXR orthologue β
COS-7	Fibroblast-like cell line derived from monkey kidney tissue
CPRG	Chlorophenol red-β-D-galactopyranoside
CV	Coefficient of variance
CYP	Cytochrome P450 enzyme
DBD	DNA-binding domain

d_N/d_S	Ratio of non-synonymous substitutions per non-synonymous site (d_N) to synonymous substitutions per synonymous site (d_S)
D_T	Tajima's D
EC	Emerging contaminants
EC_{50}	Mean effective concentration
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
ERE	Estrogen receptor response element
EST	Expressed sequence tag
FRET	Fluorescence resonance energy transfer
GAL4	Yeast DNA-binding transcription factor
GAL4-DBD	GAL4 DNA-binding domain
GM	Growth media
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
HeLa	Human cervical cancer-derived cell line
HepG2	Human liver carcinoma-derived cell line
HRV	Human rhinovirus
HSP90	Heat shock protein 90
HTS	High-throughput screening
HuH7	Human hepatocyte carcinoma-derived cell line
kb	Kilo base
LB	Luria broth

LBD	Ligand-binding domain
MCF-7	Human breast cancer-derived cell line
MCS	Multiple cloning site
MM	Minimal media
MRP	Multi-drug resistance-associated protein
NCBI CD	National Center for Biotechnology Information Conserved Domain database
NR	Nuclear receptor
NR1I	Nuclear receptor sub-family 1, class I
NR1J	Nuclear receptor sub-family 1, class J
π	Nucleotide diversity
PAHs	Polycyclic aromatic hydrocarbons
PCN	Pregnenolone 16α-carbonitrile
PCR	Polymerase chain reaction
Pfam	Protein Families database
PTX-2	Pectenotoxin-2
PTX-11	Pectenotoxin-11
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
QSAR	Quantitative structure–activity relationship
RXR	Retinoid X receptor
SAM	Seeded assay medium
SMART	Simple Modular Architecture Research Tool
SNP	Single nucleotide polymorphism

SRC-1	Steroid receptor co-activator 1
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TGF-β	Growth factor beta super-family
Ti/Tv	Ratio of transitions (Ti) versus transversions (Tv)
UAS	Upstream activation sequence
VDR	Vitamin D receptor
θ_W	Watterson's Theta
VP16	*Herpes simplex* virion protein 16
VP16-AD	VP16 activation domain
yEGFP	Yeast-enhanced green fluorescence protein

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CHAPTER ONE

Molecular evolution and functional characterisation of tunicate xenobiotic receptors and their use as sensor elements in yeast bioassays for bioactive compounds: an overview

1.1 Introduction

Bacterial, fungal and microalgal organisms in marine ecosystems collectively generate a wide range of 'bioactive' compounds that can have far-reaching effects on biological and ecological processes (e.g. reproduction, species interactions, population dynamics) as well as on human health and economies (Hay 2009; Landsberg *et al.* 2009; Mayer 2009). Metazoans have developed specialised biochemical pathways that metabolise and eliminate potentially toxic chemicals (xenobiotics) from their bodies and therefore minimise any associated deleterious effects (Li *et al.* 2007; Testa *et al.* 2012; Zanger & Schwab 2013). Xenobiotic receptors, a specific group of ligand-activated nuclear receptors (NRs), bind a structurally diverse range of xenobiotics and then activate genes involved in ligand detoxification (Nakata *et al.* 2006; Chai *et al.* 2013).

From an evolutionary perspective, xenobiotic receptors represent a highly-derived branch of the NR family tree— the root of which lies deep at the base of animal phylogeny (Bertrand *et al.* 2004). Vertebrate xenobiotic receptor proteins (pregnane X receptor, PXR) are unusual amongst the NRs as they display considerable inter-species sequence variation in the functional domain responsible for ligand-binding (ligand-binding domain, LBD; Moore *et al*. 2002; Zhang *et al*. 2004; Krasowski *et al*. 2005a, b). Such inter-taxa PXR LBD sequence differences may reflect adaptive evolutionary changes which enhance PXR binding of dietary bioactives/toxins typically encountered by an organism

(Moore *et al.* 2000; Moore *et al.* 2002; Krasowski *et al.* 2005a, b; Krasowski *et al.* 2011a, b).

Marine invertebrate filter-feeders (e.g. Tunicata, Bivalvia) are exposed to a myriad of dietary xenobiotics at relatively high concentrations (Haberkorn *et al.* 2011; Echevarria *et al.* 2012). Tunicates (phylum Chordata, subphylum Urochordata; Satoh *et al.* 2014) are of particular interest because their genomes encode putative xenobiotic receptor genes that are orthologues to vertebrate PXR (Ekins *et al.* 2008). Because tunicates accumulate high concentrations of dietary xenobiotics, tunicate xenobiotic receptors may have adaptively evolved to enhance binding of these compounds (Fidler *et al.* 2012). Tunicates are the closest living relatives of vertebrates as they are members of a sister clade to the Vertebrata (Delsuc *et al.* 2006; Delsuc *et al.* 2008). Therefore, they are a promising group to target for the development of bioassays that could be used to detect bioactive chemicals that affect vertebrate physiology.

The aims of this thesis were to: (i) determine if adaptive evolution is occurring in three tunicate putative xenobiotic receptor genes; (ii) characterise the function of two tunicate putative xenobiotic receptors using a yeast-based expression system and; (iii) develop highly generic recombinant yeast bioassays using tunicate xenobiotic receptors as sensor elements, with the goal of using these for bioprospecting and to detect harmful environmental contaminants.

1.1.1 Dietary bioactive chemicals

Chemicals in animal diets are often viewed as simply energy sources (e.g. carbohydrates, lipids), building blocks (e.g. proteins) or biochemical pathway intermediates (e.g. vitamins). However, it is apparent that some dietary chemicals can alter animal biochemistry and physiology with biological effects ranging from influences on reproduction and development through to acute poisoning (Targett & Arnold 2001; Raubenheimer & Simpson 2009; Forbey *et al.* 2013; Ortiz-Ramirez *et al.* 2013). Some animal taxa exposed to bioactive dietary xenobiotics have evolved both behavioural and physiological traits to minimise any associated deleterious effects (Dearing *et al.* 2005). Many animals

simply avoid eating plants/prey likely to contain toxins with these avoidance behaviours being both instinctual and learnt (Marsh *et al.* 2006). For example, avoidance of toxic/unpalatable prey by coral reef fish is well-documented (Long & Hay 2006; Miller & Pawlik 2013). Bivalve molluscs can limit their exposure to toxic compounds using behavioural responses such as shell closure and restriction of filtration rate (Hegaret *et al.* 2007; Haberkorn *et al.* 2011). Despite such avoidance behaviours, the diet of many marine organisms, especially those of filter-feeding and surface-grazing invertebrates (Figure 1.1) will inevitably contain toxic bioactive chemicals that need to be metabolised and eliminated from their bodies (Glendinning 2007; Fernandez-Reiriz *et al.* 2008; Manfrin *et al.* 2012; Sotka & Gantz 2013).

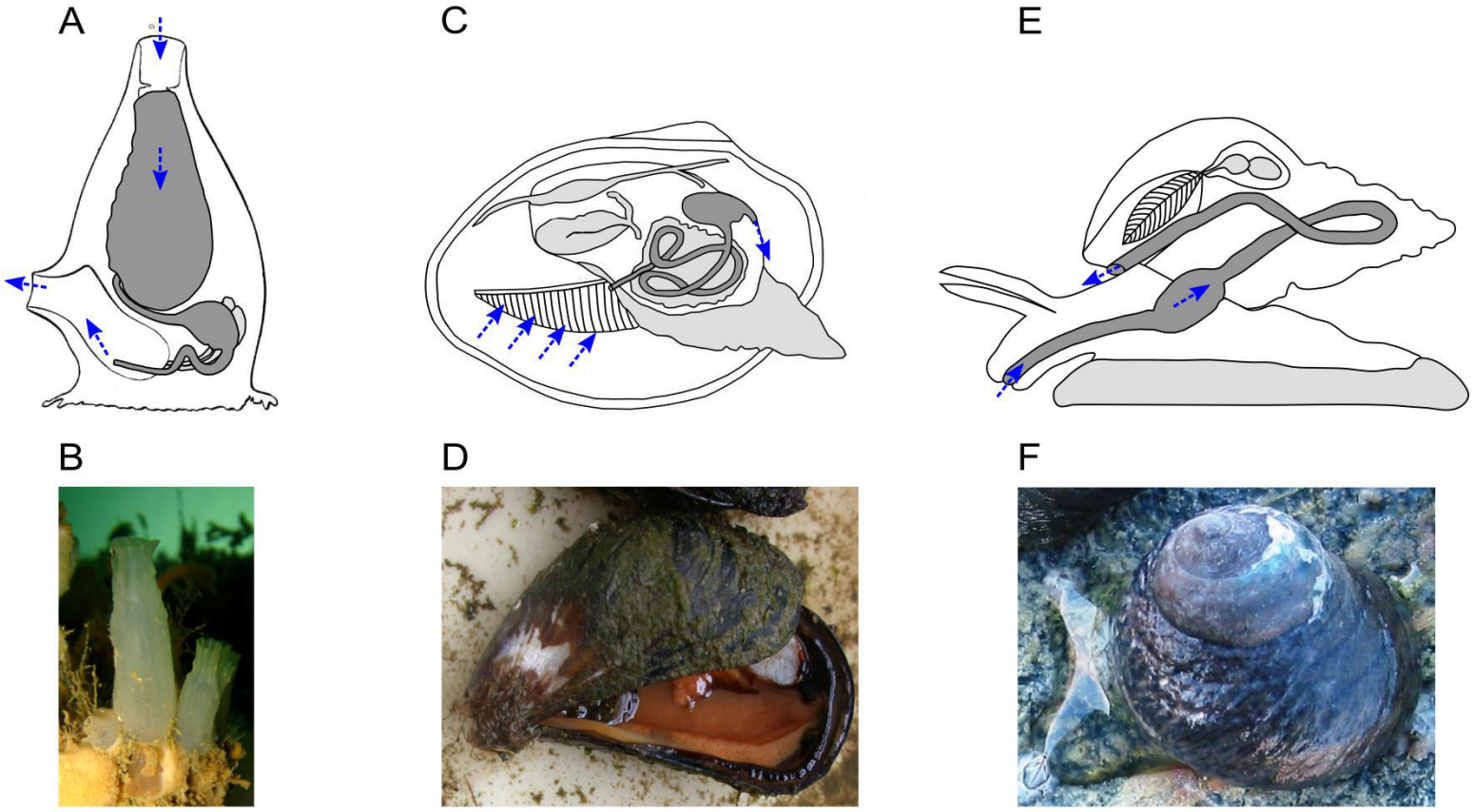

Figure 1.1 Examples of filter-feeding and surface-grazing marine invertebrates.

Schematic diagrams of the digestive tracts (dark grey) of filter-feeding tunicates (**A, B**), filter-feeding bivalve molluscs (**C, D**), and grazing gastropod molluscs (**E, F**) are shown. The direction of food movement is indicated by blue arrows. Examples of a filter-feeding tunicate (**B**), *Ciona intestinalis*, Phylum Chordata; a filter-feeding bivalve mollusc (**D**), *Mytilus edulis*, Phylum Mollusca; and a grazing gastropod mollusc (**F**), *Amphibola crenata*, Phylum Mollusca. Photos were provided by Chris Woods (**B**; National Institute of Water and Atmospheric Research, New Zealand) and Rod Asher (**D**, **F**; Cawthron Institute, New Zealand).

1.1.2 Detoxification pathways and their transcription level regulation

1.1.2.1 Metazoan detoxification pathways

Metazoan organisms have specialised biochemical pathways to metabolise and eliminate potentially toxic chemicals from their body (i.e. xenobiotics; Li *et al*. 2007; Testa *et al*. 2012; Zanger & Schwab 2013). Xenobiotic detoxification is accomplished by a number of enzymes with broad substrate specificities that are involved in one of the three phases of detoxification: oxidation/reduction (Phase I), conjugation (Phase II) and transport/elimination (Phase III; Figure 1.2; Yang *et al*. 2010; Testa *et al*. 2012).

1.1.2.2 Induction of Phase I enzymes— vertebrates

Many detoxification pathway genes can be induced by the xenobiotic(s) that the pathway ultimately metabolises (Xu *et al.* 2005; Yang *et al.* 2010; Testa *et al.* 2012; James & Ambadapadi 2013). Such xenobiotic-mediated induction of detoxification gene expression is best characterised for Phase I cytochrome P450 (CYP) enzymes, particularly members of CYP sub-families 1–4 that are associated with xenobiotic metabolism (Yamazaki 2000; Pavek & Dvorak 2008; Zanger & Schwab 2013). For example, levels of human CYP3A4, an enzyme responsible for oxidising greater than 50% of medicinal drugs, are induced by a range of therapeutic compounds such as rifampicin, tamoxifen and hyperforin (Thummel & Wilkinson 1998; Zanger *et al.* 2008). There exists inter-taxa variation in inductive responses to some xenobiotics (Martignoni *et al.* 2006). The steroidal drugs pregnenolone 16α-carbonitrile (PCN) and dexamethasone are highly efficacious CYP3A enzyme inducers in rodents, but not in humans (Martignoni *et al.* 2004; Vignati *et al.* 2004). In contrast, rifampicin is a strong inducer of human and dog CYP3A, but not of rodent CYP3A (Kocarek *et al.* 1995; Lu & Li 2001). This inter-taxa variation in the response to xenobiotics suggests the possibility of adaptive evolution in the genetic elements that control expression of detoxification genes.

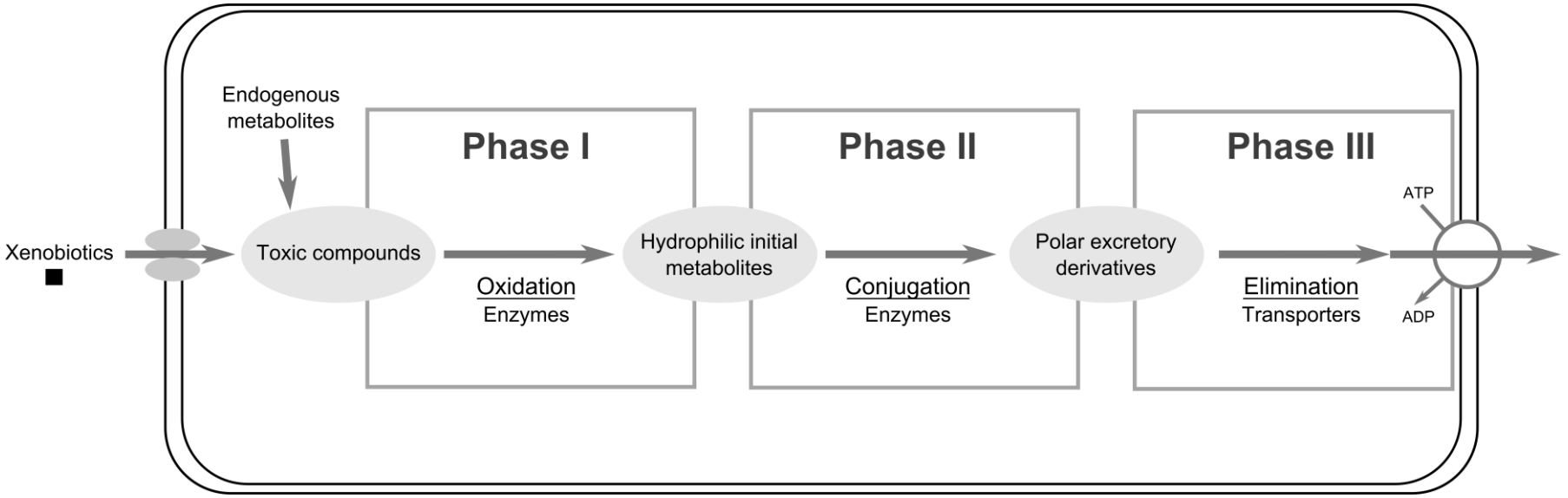

Figure 1.2 Schematic overview of metazoan detoxification pathways.

Xenobiotic detoxification is mediated via Phase I and II drug metabolising enzymes, and Phase III membrane transporters. Figure was modified from Nakata *et al*. (2006).

1.1.2.3 Induction of Phase I enzymes— invertebrates

Xenobiotic-mediated induction of CYP enzyme levels has also been reported in several invertebrate phyla, especially in the Arthropoda within the context of pesticide resistance (Rewitz *et al.* 2006; Feyereisen 2011; Schuler & Berenbaum 2013). In both dipteran and lepidopteran insect taxa, phenobarbital induces CYP enzymatic activity in association with transcription level induction of CYP4, CYP6 and CYP9 orthologues (Fisher *et al.* 2003; Natsuhara *et al.* 2004; Morra *et al.* 2010; Tomita *et al.* 2010). In the honey bee (*Apis mellifera*), aflatoxin and propolis induce CYP gene expression (Johnson *et al.* 2012). Amongst marine invertebrates, the polychaete *Perinereis nuntia* (phylum Annelida) shows increased levels of some CYP gene transcripts after exposure to benzo[α]pyrene (BaP) and polycyclic aromatic hydrocarbons (PAHs; Zheng *et al*. 2013). The marine gastropod *Cyphoma gibbosum* (phylum Mollusca) is suggested to have adapted to feeding exclusively on highly toxic gorgonian corals by differential regulation of transcripts encoding two novel CYP enzymes, CYP4BK and CYP4BL (Whalen *et al.* 2010a).

1.1.2.4 Induction of Phase II and III enzymes

While most xenobiotic-mediated gene induction research has focused on Phase I CYP genes, Phase II glutathione S-transferases (GSTs) and Phase III multi-drug resistance-associated proteins (MRPs) have also been reported to be inducible by some xenobiotics (Oakley 2011; Bousova & Skalova 2012). For example, expression of mouse GSTA1, MRP2 and MRP3 genes is induced by both PCN and 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP; Maglich *et al*. 2002). In the marine environment, dietary toxins (e.g. cyclopentenone prostaglandins) have been shown to be both inducers and substrates of GST enzymes in three marine mollusc taxa (Kuhajek & Schlenk 2003; Whalen *et al.* 2008; Whalen *et al.* 2010b).

1.1.3 Xenobiotic receptors

1.1.3.1 Overview of xenobiotic receptors belonging to the nuclear receptor super-family

It is likely that xenobiotic-mediated control of detoxification pathway gene expression may adaptively evolve in response to the various chemicals that different animals are exposed to. Diet is probably the main route of exposure to xenobiotics (Targett & Arnold 2001; Raubenheimer & Simpson 2009; Forbey *et al.* 2013). Metazoan genomes encode a range of sensors to detect xenobiotic chemicals: for example, G protein-coupled receptors that mediate taste and olfaction (Palmer 2007; Kinnamon 2012). Xenobiotic detection pathways operating independently of the nervous system are coordinated through xenobiotic receptors, a specific group of ligand-activated nuclear receptor (NR) transcription factors (Nakata *et al.* 2006; Chai *et al.* 2013; Wallace & Redinbo 2013).

Metazoan genomes encode at least three xenobiotic receptors that regulate detoxification: aryl hydrocarbon receptor (AhR), and the two NRs constitutive androstane receptor (CAR; NR notation: NR1I3) and pregnane X receptor (PXR; NR notation: NR1I2; Nakata *et al*. 2006; Chai *et al*. 2013; Wallace & Redinbo 2013). As is typical of NRs, the PXR and CAR protein structures consist of several conserved functional domains (Figure 1.3). The most conserved one is the DNA-binding domain (DBD), which is responsible for the interaction between the NRs and the response elements present in the promoter region of their target genes; and the ligand-binding domain (LBD), which interacts with the ligands and with co-regulators to control gene expression (Gronemeyer *et al.* 2004; Pascussi *et al.* 2008). Based on these highly conserved domains, seven NR sub-families (NR0–6) were defined using phylogenetic analyses (Nuclear Receptors Nomenclature Committee 1999).

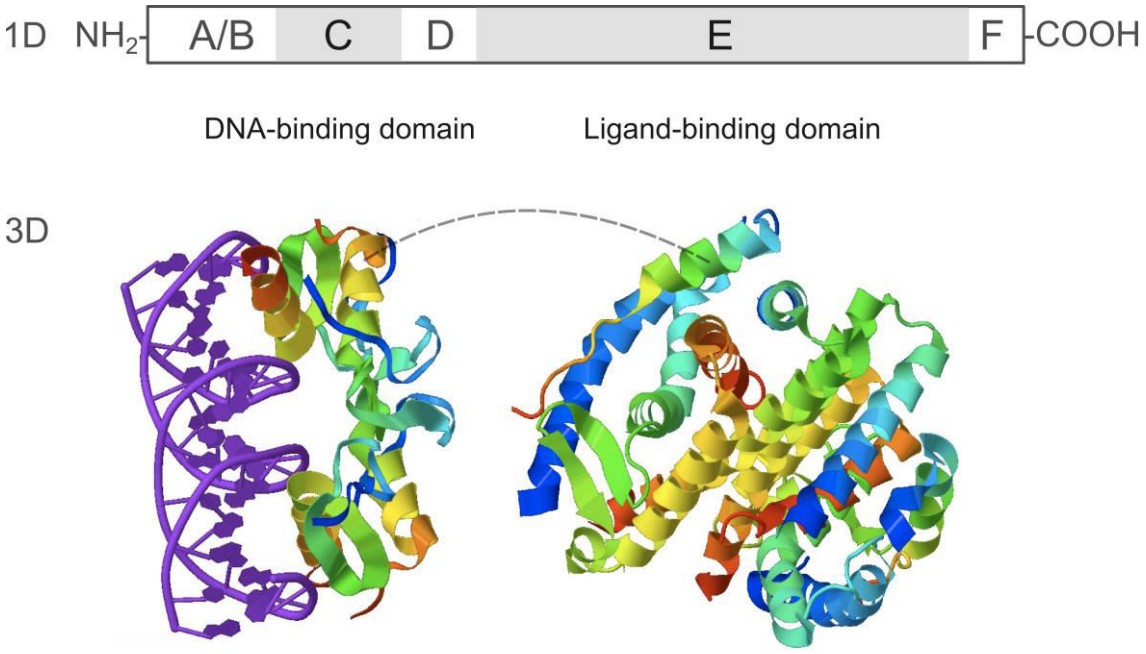

Figure 1.3 Schematic structure of nuclear receptors.

The overall structure of ligand-activated nuclear receptors is conserved through evolution with five key structural domains: N-terminal transcription activation domain (activation function 1, AF-1), DNA-binding domain (DBD), flexible hinge region (Hinge) and ligand-binding domain (LBD) which includes a C-terminal activation domain (activation function 2, AF-2). Figure was modified from Chai *et al.* (2013).

Technically, AhR is not a member of the NR super-family and instead belongs to the family of basic helix-loop-helix transcription factors (Kewley *et al.* 2004; Fujii-Kuriyama & Kawajiri 2010). This receptor consists of four major domains, some of which are similar to that of the canonical domain composition of NRs (Figure 1.3), including a DBD and LBD (Fukunaga *et al.* 1995). Activated by a wide range of xenobiotic compounds, AhR is believed to act as a detection mechanism, sensing potentially toxic foreign compounds and facilitating their eventual metabolism and elimination (Denison & Nagy 2003).

Pregnane X receptor and CAR are the master xenobiotic sensors that bind a variety of ligands including endogenous and exogenous (i.e. xenobiotic) chemicals (Chai *et al.* 2013; Xie & Chiang 2013). Although both CAR and PXR modulate a number of drug-metabolising enzymes (Kliewer *et al.* 2002; Kachaylo *et al.* 2011; Wallace & Redinbo 2013), PXR is better understood with

respect to how its LBD structure relates to ligand-binding and subsequent transcriptional activation (Chai *et al.* 2013). Crystallographic studies revealed that CAR, although promiscuous, is modulated by a smaller variety of ligands compared to the plethora that activate PXR (Wallace & Redinbo 2013).

1.1.3.2 Vertebrate pregnane X receptor

Pregnane X receptor is well characterised within a number of vertebrate taxa (Reschly & Krasowski 2006; Bainy *et al.* 2013; Chai *et al.* 2013; Xie & Chiang 2013). Vertebrate PXR was originally identified from genomic sequence data and designated as an orphan NR as its ligand(s) were then unknown (Kliewer *et al.* 1998). In 1998, three groups independently reported mammalian PXR activation by both steroids and a range of xenobiotics resulting in three alternative receptor names with PXR now being the most widely used (Bertilsson *et al.* 1998; Blumberg *et al.* 1998; Kliewer *et al.* 1998). Pregnane X receptor appears to function much like a standard ligand-activated NR. After ligand-binding within the PXR LBD, the activated PXR protein forms a complex with retinoid X receptor (RXR) before translocating from the cell cytoplasm into the nucleus. The PXR/RXR heterodimer binds to appropriate DNA response elements, thereby influencing transcription of adjacent genes (McKenna *et al.* 1999; Li & Chiang 2005; Orans *et al.* 2005; Teotico *et al.* 2008). Many of the PXR-regulated genes are involved in detoxification. Thus, PXR activation, following xenobiotic-binding to its LBD, provides a mechanistic link between the presence of xenobiotics in a cell and appropriate detoxification gene expression (Tolson & Wang 2010).

Vertebrate PXR ligands include a structurally diverse range of endogenously produced molecules; e.g. bile acids, steroid hormones and vitamins along with exogenously acquired chemicals; e.g. both synthetic and herbal drugs (Chang & Waxman 2006; Staudinger *et al.* 2006; Manez 2008; Biswas *et al.* 2009; Hernandez *et al.* 2009; Zhou *et al.* 2009). Determination of the three dimensional structure of the human PXR protein has helped explain its striking permissiveness with respect to the differing structures of activating ligands

(Wallace & Redinbo 2013; Wu *et al.* 2013). In the majority of NRs, the LBD cavities have well-defined shapes with restricted mobility, thereby ensuring specificity of ligand–LBD interactions (Eick *et al.* 2012; Harms *et al.* 2013). In contrast, the human PXR LBD is larger than is typical of NRs and displays significant flexibility during ligand-binding allowing it to accommodate a wider range of ligand sizes and structures (Wallace & Redinbo 2013; Wu *et al.* 2013).

1.1.3.3 Tunicate xenobiotic receptors

Although xenobiotic receptor encoding genes have been identified in a growing number of publicly-available invertebrate genomic sequences (Bracken-Grissom *et al.* 2014), identification of marine invertebrate xenobiotic receptors is limited to a few selected taxa (Dehal *et al.* 2002; Reitzel & Tarrant 2009; Bridgham *et al.* 2010; Denoeud *et al.* 2010; Srivastava *et al.* 2010; Vogeler *et al.* 2014). The first putative xenobiotic receptor from a marine invertebrate was identified in the genome of the solitary tunicate *Ciona intestinalis,* which was also the first marine invertebrate to have an assembled and annotated genome published (Dehal *et al.* 2002; Satou *et al.* 2002; Yagi *et al.* 2003). Analysis of the *C. intestinalis* genomic sequence, in combination with *C. intestinalis* expressed sequence tag (EST) databases, revealed two genes that phylogenetic analyses placed as orthologues to vertebrate NR1I genes. These two *C. intestinalis* NR1I-like genes were equally related to the vertebrate PXR, CAR and vitamin D receptor (VDR; NR notation: NR1I1) genes, hence denoted *Ci*VDR/PXRα (GenBank accession number: NM_001078379) and *Ci*VDR/PXRβ (NM_001044366; Dehal *et al*. 2002; Yagi *et al*. 2003; Satou *et al*. 2005). More recently, two putative PXR/NR1I orthologues have been detected in the genomic sequence of the colonial tunicate *Botryllus schlosseri* (Voskoboynik *et al.* 2013), while the genome of the pelagic tunicate *Oikopleura dioica* encodes as many as six NR1I clade genes (Denoeud *et al.* 2010).

The ligand-binding characteristics of the putative *C. intestinalis* PXR orthologue were subsequently investigated using a mammalian expression system (Reschly *et al.* 2007; Ekins *et al.* 2008). The *Ci*VDR/PXRα LBD was fused to the

DBD of the generic yeast transcription factor GAL4 (GAL4-DBD) and the resulting chimeric protein was expressed in a mammalian cell line carrying a ligand-dependent luciferase reporter gene (Reschly *et al.* 2007; Ekins *et al.* 2008). Using this bioassay, three synthetic chemicals (6-formylindolo-[3,2-b]carbazole, *n*-butyl-*p*-aminobenzoate and carbamazepine) and two microalgal biotoxins (pectenotoxin-2 (PTX-2) and okadaic acid) were identified as *Ci*VDR/PXRα LBD agonists (Reschly *et al.* 2007; Ekins *et al.* 2008; Fidler *et al.* 2012). A common pharmacophore model of *Ci*VDR/PXRα activators was tentatively defined. The model consists of a planar structure with at least one off-centre hydrogen bond acceptor flanked by two hydrophobic regions (Ekins *et al.* 2008; Fidler *et al.* 2012). Both microalgal biotoxins (okadaic acid and PTX-2) were two to three orders of magnitude more potent than the three synthetic ligands. This supports the idea that the natural *Ci*VDR/PXRα receptor is activated by exogenous compounds relevant to its marine environment, thus playing a similar role in detecting and eliminating xenobiotics as its vertebrate orthologue (Sladek 2011; Fidler *et al.* 2012). This assumption is supported by the following observations: (i) putative endogenous ligands (e.g. vitamin D or bile salt correlates) have not yet been identified in invertebrates (Reschly *et al.* 2007; Ekins *et al.* 2008) and; (ii) multiple canonical xenobiotic response elements are present in the upstream genomic sequence of most *CYP1* genes (CYP1A- and CYP1B/CYP1C-like) in *C. intestinalis* (Goldstone *et al.* 2007).

1.1.4 Suitable expression systems for functional studies of nuclear receptors

Several *in vitro* methods can be used for the functional characterisation of orthologues genes from different organisms, outside of the context of a whole, intact organism (Jensen *et al.* 2007). Recombinant proteins can be generated in bacteria, in cultured eukaryotic cells or in a cell-free system, and assayed for activity of specified substrates. While such *in vitro* methods tend to be faster and cheaper than studies on whole organisms, there might be only an indirect connection between *in vitro* receptor function and organismal phenotypes (Jensen *et al.* 2007). However, using mammalian cell line-based expression

systems, NR ligands have been successfully identified and correlated to results obtained *in vivo* (Luo *et al.* 2002; Sinz *et al.* 2006; Chu *et al.* 2009).

1.1.4.1 Mammalian cell lines for functional studies of nuclear receptors

Mammalian cell line-based reporter assays, as described in Section 1.1.3.3, have been used extensively for the functional characterisation of PXR, CAR and AhR (Raucy & Lasker 2013). The main advantage of mammalian cell lines is that they share a similar cellular/biochemical milieu with metazoan cells, which is expected to assist correct folding and functioning of NR proteins expressed from heterologous genes. Using mammalian cell lines, receptor ligands/activators can be identified using ligand-binding assays or cell-based transfection assays (Raucy & Lasker 2013).

One popular approach for reporter assays is to make chimeric receptors containing the LBD of the receptor of interest fused to the GAL4-DBD. The reporter plasmids are then designed to respond to GAL4 (Stanley *et al.* 2006). This approach identified different compounds as potent activators of both the human and mouse PXR; using a GAL4-luciferase reporter construct responding to human PXR-LBD/GAL4-DBD or mouse PXR-LBD/GAL4-DBD chimeric receptors in HepG2 cells (Vignati *et al.* 2004). The use of a single reporter construct allows for direct comparisons of receptor–ligand interactions. However, the use of chimeric receptors means that subtle conformational changes and protein–protein interactions mediated by regions other than the LBD of the receptor of interest may be lost. For example, TCPOBOP only activated the full-length mouse CAR, but not the mouse CAR-LBD/GAL4-DBD chimeric protein. This suggests that the full-length receptor constructs represent the *in vivo* situation better than the GAL4-based assay (Moore *et al.* 2002).

To overcome these limitations, transactivation of the full-length NRs can be assessed in either transient or stable cell lines. In transient cell-lines, the transfected genetic material is not integrated into the nuclear genome. Thus, the foreign DNA is transiently expressed because it will be degraded or diluted via mitosis. In stable cell lines, a fraction of the transfected genetic material is

introduced into the genome. The most common method involves transient transfection of the receptor and a response element-reporter gene construct. With this approach, the full-length human PXR, human CAR or human AhR cDNAs are cloned into an expression vector. In addition, chimeric luciferase, β-lactamase, or chloramphenicol acyl transferase reporter vectors are prepared containing the appropriate NR response elements (Stanley *et al.* 2006). Using transient transactivation systems, several studies have identified human PXR and AhR activators, and correlated these results to those obtained *in vivo* (Luo *et al.* 2002; Sinz *et al.* 2006; Chu *et al.* 2009). A number of disadvantages are associated with transient transfection assays including the inherent variability associated with transfection efficiency and the time required to perform these assays (Raucy & Lasker 2013).

1.1.4.2 Recombinant yeast strains for functional studies of nuclear receptors

A valuable alternative expression system is the well-characterised unicellular eukaryotic organism *Saccharomyces cerevisiae* (common baker's yeast). Yeast is easy to cultivate and there are numerous tools available for its genetic manipulation. Due to some common characteristics with mammalian cells, yeast has become a popular organism in studying NR functions and ligand affinities (Chen *et al.* 2004; Fox *et al.* 2008; Balsiger *et al.* 2010; Miller *et al.* 2010; Raucy & Lasker 2010; Rajasarkka & Virta 2011; Li *et al.* 2013). Some of the resulting yeast strains have found application in screening environmental samples for bioactivities — particularly for estrogenic activity (Routledge & Sumpter 1996; Collins *et al.* 1997; Gaido *et al.* 1997; Chatterjee *et al.* 2008; Passos *et al.* 2009; Balsiger *et al.* 2010; Chen *et al.* 2014). Finally, *S. cerevisiae* offers the possibility of directed evolution of NRs. *In vitro* mutagenesis can be used to generate NR LBD variant sequences, which can be selected for enhancement of growth rates in the presence of a cognate ligand (Chen & Zhao 2003).

Despite the clear advantages of recombinant NR-based yeast bioassays, these cell-based bioassays face a number of limitations. Such limitations include the

inability to express structurally complex proteins. Members of the growth factor beta (TGF-β) super-family, important proteins involved in cell growth, cell differentiation, apoptosis and cellular homeostasis, were only expressed successfully as recombinant proteins in mammalian expression systems (Kim *et al.* 2002). Limitations intrinsic to all living cells include elimination of potential NR ligands/test compounds via yeast export pumps (Liu *et al.* 1999; Dudley *et al.* 2000). It was shown that by removing such transporters from the yeast genome, the sensitivity of yeast bioassays can be improved (Balsiger *et al.* 2010). Low permeability of test compounds through the yeast cell wall and membrane can limit assay sensitivity (Lyttle *et al.* 1992), although NR-based yeast bioassays were shown to be highly sensitive even with very short incubation times (4–12 hours; Bovee *et al.* 2004).

A number of yeast systems were developed to detect macromolecular interactions: DNA–protein (one-hybrid assay), protein–protein (two-hybrid assay) and RNA–protein (three-hybrid assay; Mager & Winderickx 2005; Vidal & Legrain 1999). The yeast two-hybrid system represents a powerful approach for detecting protein–protein interactions in cells and, more recently, has evolved from the original yeast two-hybrid system into a method for identifying NR ligands (reviewed in Fox *et al.* 2008).

The 'yeast estrogen screen' is an elegant example of a modified one-hybrid method designed to identify small-molecule compounds that bind to heterologous NR proteins expressed in yeast. The human estrogen receptor alpha (ERα) is integrated into the main yeast chromosome. The yeast cells also contain an expression plasmid carrying the reporter gene *lacZ* which encodes the enzyme β-galactosidase (Figure 1.4A; Routledge & Sumpter 1996; Bovee *et al.* 2004). The ligand-bound ER binds the estrogen receptor response element (ERE) inserted on the promoter of the *lacZ* reporter gene. Then transcriptional activation of the *lacZ* reporter takes place via recruitment of various yeast transcription cofactors (e.g. chromatin remodelling complexes or histone modification factors; Kennedy 2002).

In this approach, the native, full-length NR is being used to identify potential NR ligands. Thus, both the NR coactivators and the sequences of the cognate DNA elements to which the NR DBD binds need to be known. Such specialised

knowledge may not be available for some NRs, especially in the case of orthologues for invertebrate xenobiotic receptors. Even though putative response elements for metazoan xenobiotic receptors have been identified in marine invertebrate genomes (Goldstone *et al.* 2007), these may not function as required in *S. cerevisiae* cells.

These knowledge gaps can be bypassed by exploiting the highly modular structure of NRs (Figure 1.3). A chimeric protein can be generated in which the LBD of interest is fused to the GAL4-DBD. These transcription factors contain: (i) modular DBDs that bind to specific DNA response elements and; (ii) modular transcriptional activation domains (ADs) that interact with the basal transcription machinery to increase transcriptional activation (Figure 1.4B; Raucy & Lasker 2010). The interaction between the NR LBD and a ligand is detected using a reporter gene containing well-characterised DNA control elements (e.g. GAL4 upstream activation sequence, UAS; Young 1998). This removes the need for knowledge of the natural heterodimer partners of the NR or the DNA sequence elements to which the NR binds through its native DBD. Following binding to a well-characterised UAS element, via the GAL4-DBD, the NR's ligand-dependent activation domain (AF-2, Figure 1.3) needs to function within the nuclear milieu of yeast cells. As previous studies have shown that the AF-2 domains of some vertebrate NRs do not function in yeast cells (Berry *et al.* 1990; Louvion *et al.* 1993), a generic transcription activation domain from the *Herpes simplex* virion protein 16 (VP16-AD) can be added to the C-terminus of the chimeric proteins (Louvion *et al.* 1993). In summary, a fusion gene can be generated encoding a chimeric protein that contains the GAL4-DBD, the NR LBD and the VP16-AD, with the ligand-binding characteristics of the chimeric protein determined by the NR LBD (Figure 1.4B).

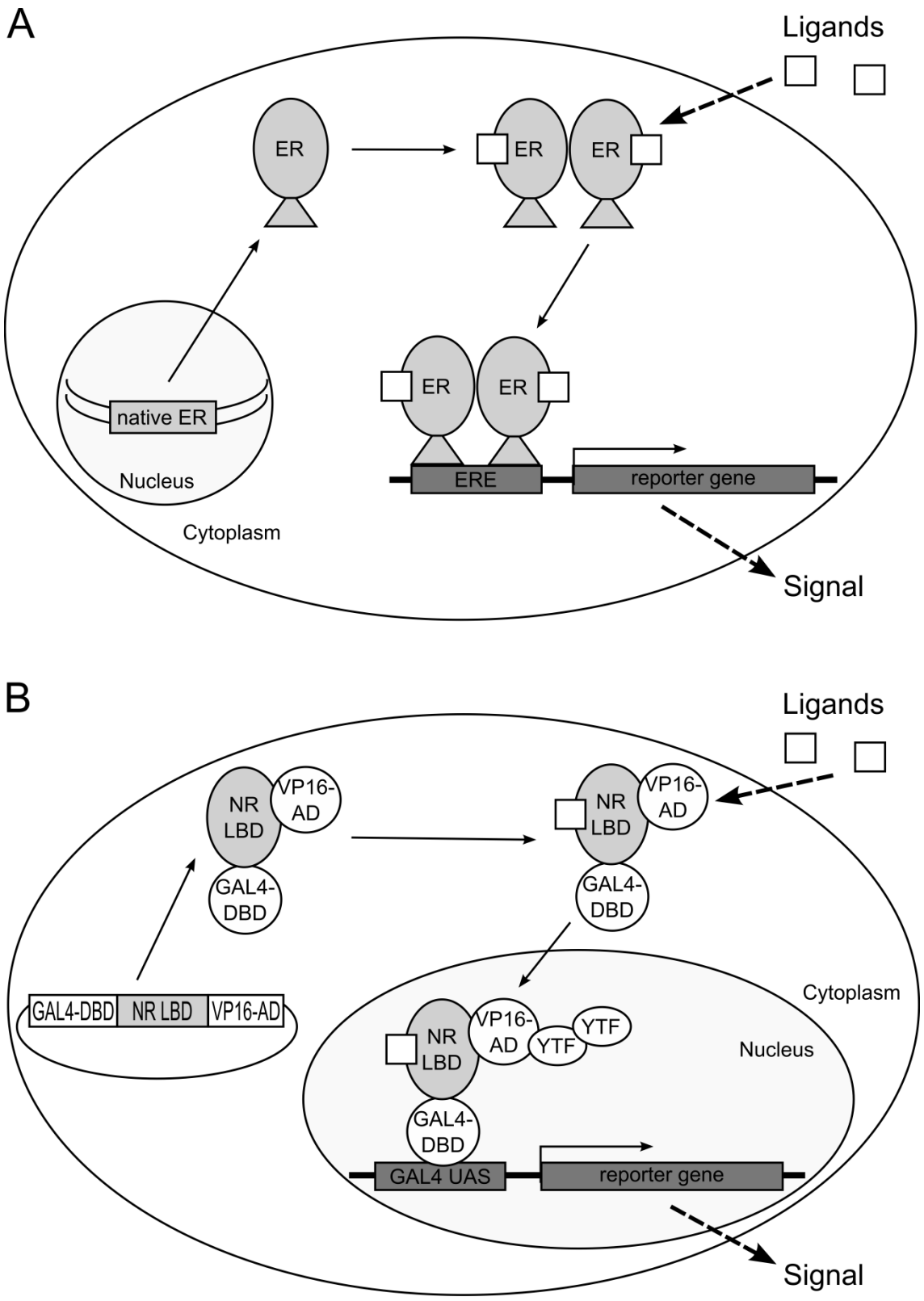

Figure 1.4 Simplified schematic of modified yeast one-hybrid bioassays.

(**A**) In the yeast estrogen assay the native human estrogen receptor alpha (ERα) is integrated into the main genome and is expressed in a form capable of binding estrogen response elements (ERE) within a hybrid promoter on the expression plasmid

encoding the reporter gene. (**B**) In a modified yeast one-hybrid assay, a yeast host strain has a plasmid carrying a chimeric gene encoding a fusion protein containing the DNA-binding domain (DBD) of the yeast transcription factor GAL4 (GAL4-DBD), a nuclear receptor (NR) ligand-binding domain (LBD) and a generic activation domain (AD) from the viral protein VP16 (VP16-AD). Ligand-binding leads to a conformational change of the chimeric protein, resulting in binding of the GAL4-DBD to the control region in the promoter region of the reporter gene which is integrated in the yeast strain genome. The VP16-AD is responsible for recruitment of general yeast transcription factors (YTF).

The reporter gene selected to generate the 'output' signal from yeast bioassays must combine low background with a clear response signal following activation of the NR LBD. Three types of NR-dependent reporter gene assays have been used in recombinant yeast: the *Escherichia coli lacZ* gene, encoding the enzyme β-galactosidase (de Almeida *et al.* 2008), yeast-enhanced green fluorescence protein (yEGFP; Bovee *et al*. 2007; Chatterjee *et al*. 2008; Bovee *et al*. 2011) and the luciferase gene (Nordeen 1988). Although the luciferase and yEGFP reporter assays are more sensitive than *lacZ* (Fan & Wood 2007), both have associated complications. Luciferase assays require the use of expensive substrates and involve cell lysis (Hancock *et al.* 2007; de Almeida *et al.* 2008) which can be problematic either due to released cellular proteases (de Almeida *et al.* 2008) or incomplete cell lysis (Hancock *et al.* 2007). Although yEGFP assays do not require the addition of substrates, the assays are characterised by a high natural background of green fluorescence (Bovee *et al.* 2007). In contrast, *lacZ* assays are inexpensive and, when based on the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG), do not require cell lysis (Routledge & Sumpter 1996). Such non-lethal measurements of β-galactosidase activity are useful as it means that repeated measurements can be taken over time. This is a significant advantage because the time course of *lacZ* gene transcription induction will vary between ligands due to differences in parameters such as membrane permeability and solubility in the cytoplasm (Norcliffe *et al.* 2013).

1.2 Thesis structure and research questions

This thesis aims to build on and extend current advances in the molecular evolution and function of a specific class of transcription regulating proteins— the xenobiotic receptors— from marine invertebrates (Tunicata). This thesis is divided into two parts. Part one examines the molecular evolution of tunicate xenobiotic receptors in response to dietary bioactive compounds (Chapter Two). Part two focusses on the functional characterisation of tunicate xenobiotic receptors in a yeast expression system and the subsequent application of these recombinant yeast bioassays for the detection of bioactive compounds (Chapters Three, Four and Five).

The over-arching aim of this thesis was to understand the fundamental role of tunicate orthologues of the pregnane X receptor (PXR) and vitamin D receptor (VDR) and their potential application in yeast bioassays for the detection of both bioactive compounds and environmental contaminants. This was addressed through a series of four research questions:

(i) Have tunicate VDR/PXR ligand-binding domains (LBDs) adaptively evolved to enhance binding of exogenous dietary bioactives/toxins typically encountered by these organisms?

(ii) Are tunicate VDR/PXR LBDs activated by dietary xenobiotics (e.g. microalgal biotoxins) and can these receptors be used as sensor elements in yeast bioassays for the detection of microalgal biotoxins?

(iii) Can tunicate VDR/PXR LBD-based yeast bioassays be used to detect natural bioactive compounds that represent potential drug lead compounds?

(iv) Can tunicate VDR/PXR LBD-based yeast bioassays be used to detect environmental contaminants that negatively affect a wide range of metazoan species?

Chapter One provides a general overview of the molecular evolution and functional characterisation of metazoan PXRs with a focus on marine invertebrate PXRs.

Chapter Two documents the identification of genetic variation within two PXR orthologues in the solitary tunicate *Ciona intestinalis* (designated *Ci*VDR/PXRα and β) and one PXR orthologue in the colonial tunicate *Botryllus schlosseri* (*Bs*VDR/PXRα).

Chapter Three describes the functional expression of two tunicate VDR/PXR orthologues genes (*Ci*VDR/PXRα and *Bs*VDR/PXRα) in recombinant yeast (*Saccharomyces cerevisiae*) strains.

Chapter Four extends the work described in Chapter Three by screening a wide range of natural bioactive compounds and relevant analogues for activity in the tunicate VDR/PXR LBD-based yeast bioassay.

Chapter Five discusses the potential application of the tunicate VDR/PXR LBD-based yeast bioassay in environmental monitoring to detect harmful synthetic chemicals that represent emerging contaminants.

Finally, Chapter Six provides a synthesis of the proceeding chapters and a discussion on the general implications of this work and potential future research.

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Ciona intestinalis from Lyttelton Harbour, New Zealand.
Photograph by Dr Chris Woods, National Institute of Water and Atmospheric Research, Christchurch, New Zealand.

Part One

Detection of adaptive evolution in tunicate xenobiotic receptors using molecular tools

CHAPTER TWO

Transcripts of tunicate orthologues of the pregnane X receptor: characterisation and natural variation

2.1 Introduction

Tunicates feed by filtering small organisms from seawater and therefore can be exposed to dietary xenobiotics at relatively high concentrations (Sekiguchi *et al.* 2001; Echevarria *et al.* 2012; Roje-Busatto & Ujević 2014). Dietary xenobiotics that are toxic to tunicates (i.e. increase mortality/morbidity and/or reduce reproductive output) are likely to act as agents of natural selective pressures. Ligand-binding domains (LBDs) of xenobiotic receptors may evolve to include these compounds in their repertoire of potential ligands (Fidler *et al.* 2012). Evidence of adaptive evolution has been reported within LBD sequences of vertebrate xenobiotic receptors. For example, vertebrate pregnane X receptor (PXR) displays greater inter-taxa variation in LBD sequences than is typical of nuclear receptors (NRs), along with some evidence of positive selection within the LBD (Moore *et al.* 2002; Zhang *et al.* 2004; Krasowski *et al.* 2005a, b). Tunicate xenobiotic receptors are orthologues to vertebrate vitamin D receptor (VDR), constitutive androstane receptor (CAR) and PXR, hence abbreviated VDR/PXR. The LBD of *Ciona intestinalis* VDR/PXRα has a low sequence identity when compared to vertebrate VDR, CAR and PXR (17–27% sequence identity). In contrast, the DNA-binding domain (DBD) is more conserved (60–68% sequence identity; Table 2.1). It has been speculated that such inter-taxa PXR LBD sequence differences may reflect adaptive evolutionary changes enhancing binding of exogenous dietary xenobiotics typically encountered by an organism (Moore *et al.* 2002; Zhang *et al.* 2004).

Table 2.1 Sequence identities of xenobiotic receptor orthologues.
Sequence identities of *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα) compared to the vertebrate pregnane X receptor (PXR), the vitamin D receptor (VDR) and constitutive androstane receptor (CAR). Table modified from Ekins *et al.* (2008).

Receptor	% Identity to *Ciona intestinalis* VDR/PXRα	
	DNA-binding domain	Ligand-binding domain
Human PXR	61.8	22.5
Mouse PXR	60.3	21.5
Chicken PXR	63.2	23.7
Xenopus PXR	64.7	20.3
Fugu PXR	67.6	19.8
Human VDR	67.6	17.1
Zebrafish VDR	70.6	21.8
Sea lamprey VDR	73.5	20.8
Human CAR	60.3	26.8
Mouse CAR	55.9	23.2

Characterising the effects of natural selection at the molecular level is one of the major challenges of evolutionary genomics. In general, variation at the genetic level can affect the function of proteins, which can lead to cellular modifications, organismal phenotypes and reduced fitness (Dalziel *et al.* 2009). Thus, protein adaptations are characterised by 'footprints' in the genome which can be identified in genomic sequence data and analysed using a wide range of statistical methods (Biswas & Akey 2006). McDonald and Kreitman (1991) suggested to take advantage of the degeneracy of the genetic code: non-synonymous variation (i.e. amino acid changing) is supposed to reflect the action of natural selection, whereas synonymous changes are seen as a neutral reference. For coding sequences, comparisons between the number of non-synonymous substitutions per non-synonymous site (d_N) to synonymous substitutions per synonymous site (d_S) provide a good measure of the strength and character of selection (Jensen *et al.* 2007). Using divergence-based maximum likelihood analyses, positive selection was detected in a number of highly variable sites in Mu-class glutathione transferases, a class of vertebrate detoxification enzymes (Ivarsson *et al.* 2003). These putatively selected

changes were shown to lead to differences in protein function. This is consistent with the hypothesis that functional diversification underlies rapid amino acid evolution in detoxification genes (Norrgard *et al.* 2006).

Previous suggestions of positive selection acting on the LBD coding sequences of PXR, and its orthologues, were based on sequence comparisons between vertebrate taxa (Zhang *et al.* 2004; Krasowski *et al.* 2005a). To date, inter-species studies have shown significant differences in the xenobiotic response between humans and rodents which may be explained by differences in the pharmacology of mammalian PXR (Blumberg *et al.* 1998; Kliewer *et al.* 1998; Jones *et al.* 2000; LeCluyse 2001; Tirona *et al.* 2004). The aims of this chapter were to investigate intra-species sequence variation in VDR/PXR LBD coding sequences of two tunicates, *C. intestinalis* and *Botryllus schlosseri*, and to identify evidence of both adaptive evolution and functionally significant allelic variations.

2.2 Materials and methods

2.2.1 Tunicate collection and tissue sampling

Ciona intestinalis adults (>60 mm length, n = 30) were collected from four New Zealand South Island locations: (i) Nelson marina (41°15'32.64"S, 173°16'55.53"E, n = 18); (ii) Lyttelton Harbour (43°36'38.63"S, 172°42'14.35"E, n = 3); (iii) Pelorus Sound (41°12'54.87"S, 173°52'46.91"E, n = 6) and; (iv) Queen Charlotte Sound (41°12'31.73"S, 174°17'59.91"E, n = 3). New Zealand *Botryllus schlosseri* colonies (n = 30) were collected from two locations: Nelson marina (n = 23) and Lyttelton Harbour (n = 7). *Botryllus schlosseri* colonies (n = 10) from the eastern Mediterranean Coast were collected from a single location: Michmoret Beach, Ruppin Academic Centre, Israel (32°24'31.85"N, 34°52'05.37"E). The *Botryllus schlosseri* colonies were collected >1 m apart to reduce the chances of sampling clonally related colonies. *Ciona intestinalis* individuals were kept overnight in sea water with traces of menthol crystals to

anesthetise the animals to assist subsequent dissection of tissues (Hanashima *et al.* 2012). Dissected *C. intestinalis* tissues were frozen on dry ice before storage at -70°C. *Botryllus schlosseri* colonies from New Zealand waters were frozen on dry ice before storage at -70°C. *Botryllus schlosseri* colonies collected in the Mediterranean were placed in RNA stabilisation buffer (http://sfg.stanford.edu/RNAbuffer.pdf) and transported at ambient temperatures to New Zealand before long term storage at -70°C.

2.2.2 Amplification and Sanger sequencing of cDNA sequences

Total RNA was isolated from either dissected *C. intestinalis* gut tissues or *B. schlosseri* colony fragments using Trizol (Life Technologies, Carlsbad, CA, U.S.A.) following the manufacturer's protocol. First strand cDNAs were synthesised from total RNA using random hexamer primers (Transcriptor First Strand cDNA Synthesis Kit, Roche Diagnostics, Penzberg, Germany) and then used as templates in polymerase chain reactions (PCRs). Primers were designed to amplify three tunicate VDR/PXR partial coding sequences. Primers flanking the coding sequences for the predicted DBD and LBD domains of *C. intestinalis* VDR/PXRα (*Ci*VDR/PXRα, GenBank acc. no.: NM_001078379) and *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, NM_001044366) were designed directly from the GenBank sequences with the primer sequences shown in Appendix 1A. When this work was started, sequencing of the *B. schlosseri* genome was still in progress which complicated PCR primer design. Primers for amplifying the *B. schlosseri* VDR/PXRα coding sequence were developed using a combination of alignments of PXR orthologues sequences and *B. schlosseri* expressed sequence tag (EST) sequence data. The predicted DBD protein sequences of PXR orthologues from mouse (AF031814), rat (AF151377), rabbit (AF188476), human (AF061056), frog (AF305201), chicken (AF276753) and *C. intestinalis* (NM_001078379) were aligned. A conserved peptide sequence, CEGCKGFFR, was identified and a redundant forward primer was designed from an alignment of the corresponding DNA sequences (Appendix 1A). The forward primer was paired with a reverse primer designed directly from two *B.*

schlosseri EST sequences (contig-18161978, contig-18290615, A. Voskoboynik, *pers. comm*.) identified as encoding a VDR/PXRα orthologue (Appendix 1A). Polymerase chain reactions were carried out in 20 µl final volumes containing: 10 µl of SAHARA™ DNA Polymerase 2x Master Mix (Bioline, London, U.K.), forward and reverse primers (both 0.4 µM), 32 µg/ml bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, U.S.A.) and 2 µl of template cDNA. All three partial coding sequences (*Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Bs*VDR/PXRα) were amplified using the following thermocycling conditions: 94°C/10 min., 1 cycle; 94°C/30 sec., 45°C/30 sec. ramping at 0.2°C/sec. to 72°C/2 min., 5 cycles; 94°C/30 sec., 55°C/30 sec., 72°C/2 min., 35 cycles; 72°C/7 min., 1 cycle; 15°C/hold. The resulting PCR products were visualised on an agarose gel and purified (Zymoclean™ Gel DNA Recovery Kit, Zymo Research, Irvine, CA, U.S.A.). Purified amplicons were ligated into pGEM-T-Easy™ (Promega, Madison, WI, U.S.A.), transformed into DH5α *Escherichia coli*, and plasmids purified (High Pure Plasmid Isolation Kit, Roche Diagnostics). Plasmid inserts were bi-directionally sequenced by an external contractor using a vector encoded primer pair, M13 forward and reverse (Massey Genome Service, Massey University, New Zealand). A representative sequence from a single cloned haplotype from each of the three genes was deposited in GenBank with the following accession numbers: *Ci*VDR/PXRα (1326 bp): KC561370, *Ci*VDR/PXRβ (1569 bp): KC561371 and *Bs*VDR/PXRα (1006 bp): KC561372.

2.2.3 Protein analysis software

Structural domains of predicted protein sequences were identified using the Simple Modular Architecture Research Tool (SMART) software (Letunic *et al*. 2012; http://smart.embl-heidelberg.de), the National Center for Biotechnology Information (NCBI) Conserved Domain (CD) database (Marchler-Bauer *et al*. 2011; http://ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Protein Families (Pfam) database (Punta *et al*. 2012; http://pfam.xfam.org).

2.2.4 Phylogenetic analysis

DNA-binding and ligand-binding domain sequences from predicted chordate NR1I clade proteins were aligned using ClustalW (Thompson *et al.* 1994) implemented in the MEGA5 package (Molecular Evolutionary Genetics Analysis software, version 5; Tamura *et al*. 2011). Phylogenetic analyses were performed using MEGA5 with alignment positions containing gaps/missing data eliminated from the dataset (complete deletion option). Phylogenies were inferred using the neighbour-joining algorithm with maximum composite likelihood distances and 1000 bootstrap repetitions. Sequences from domestic silkworm (*Bombyx mori*) ecdysone receptor (AAA87341), tick (*Amblyomma americanum*) ecdysone receptor (AAB94566) and sea urchin (*Strongylocentrotus purpuratus*) ecdysone receptor/farnesoid X receptor (NP_001123279) were used as outgroups.

2.2.5 Detection of *Ciona intestinalis* VDR/PXRα and β gene transcripts

Quantitative PCR (qPCR) was used to detect *Ci*VDR/PXRα and β transcripts in five regions of the *C. intestinalis* digestive tract. Total RNA was isolated from adult *C. intestinalis* digestive tract tissues using Trizol® Reagent. cDNA was synthesized as described in Section 2.2.2 and quantified using a Qubit® 2.0 Fluorometer (Life Technologies). A *Ciona intestinalis* cytoskeletal actin gene orthologue (GenBank acc. no.: AJ297725) was used as an internal control for cDNA synthesis. All qPCR primer pairs used are listed in Appendix 1B and were predicted to flank at least one intron based on a *C. intestinalis* genomic sequence (build 1.1, NCBI). Amplifications were performed in 20 µl reactions containing: 0.4 µM of both primers, 10 µl iQ™ SYBR® Green 2x SuperMix (BioRad, Hercules, CA, U.S.A.) and 2 µl template cDNA. Negative control reactions, in which sterile water replaced the cDNA, were run in parallel. The qPCR thermocycling conditions used were as follows: 95°C/2 min., 1 cycle; 95°C/15 sec., 60°C/1 min., 40 cycles (Rotor-Gene™ 6000, Qiagen, Hilden,

Germany). Amplicon homogeneity and absence of primer dimers was confirmed by melt curve analysis. Data obtained from triplicate amplifications of each template were averaged and expressed in molecules/ng cDNA using the appropriate diluted plasmid-based standard curves. Amplicons were cloned into a T-tailed vector (pGEM-T-Easy™, Promega) to provide quantifiable templates for generation of standard curves. Plasmid concentrations were measured using a Nano Photometer (Implen, Munich, Germany) and then serially diluted 1:10 in water to generate standard curves displaying a linear relationship between the cycle threshold values and log10 plasmid molecule number over the range 10^2–10^7 plasmid molecules/reaction (R^2 >0.995, M = -3.4, efficiency = 0.9). The data were square root transformed and one-way analysis of variance (ANOVA) was used to study the association between different tissues and the level of gene expression using the Tukey HSD test function in Statistica 9 (StatSoft Inc, Tulsa, OK, U.S.A., 2008). Homogeneity of the variances was tested using Cochran's C test and a *p* value <0.01 was considered significant.

2.2.6 Illumina™ sequencing of pooled amplicons

Tunicate VDR/PXR coding region amplicons were generated from either gut cDNA (*C. intestinalis*) or colony cDNA (*B. schlosseri*) templates using primers listed in Appendix 1A. Amplicons were purified from agarose gels (Zymoclean™ Gel DNA Recovery Kit, Zymo Research) and concentrations determined using a Nano Photometer (Implen). Amplicons were then pooled on an equimolar basis generating separate pooled mixtures for the *Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Bs*VDR/PXRα amplicons. The total DNA concentration in each of the amplicon mixtures was adjusted to 2 ng/µl (Nano Photometer, Implen). Subsequent sequencing of the amplicon pools was carried out by an external contractor (New Zealand Genomics Ltd., Massey University, New Zealand). The three amplicon mixes were characterised using an Agilent Bioanalyzer DNA 7500 Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and the Quant-iT™ dsDNA HS Assay Kit (Life Technologies) before pooling into a single mixture containing all three amplicons. The mixture was sheared by nebulisation and sequenced

using the Illumina™ TruSeq DNA Library Preparation Kit in combination with the Illumina™ MiSeq System (Illumina™, San Diego, CA, U.S.A.) producing paired-end reads with a mean length of ca. 150 bp. The resulting data was quality controlled using conventional procedures: (i) the Burrows-Wheeler Alignment (BWA) tool (Li & Durbin 2010) was used to map raw sequence files against the PhiX genome and all matching reads were removed; (ii) adapter removal was performed using the fastx-clipper programme implemented in the ea-utils suite (version 1.1.2-318; Aronesty 2013) and; (iii) to remove any other potential contamination the reads were screened against a set of genome sequences (e.g. *E. coli*, Illumina™ adapters and the cloning vector pFosill-2) using fastQscreen (Babraham Bioinformatics 2013a). Sequence quality statistics were calculated using fastQC (Babraham Bioinformatics 2013b) and SolexaQA (Cox *et al.* 2010). The remaining sequences were aligned against the reference sequences for each of the three amplicons in paired-end and single-end mode using the BWA tool.

2.2.7 Identification and analysis of polymorphisms in pooled amplicon sequence data

The Illumina™ MiSeq generated sequence data for the *Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Bs*VDR/PXRα amplicons was examined for variants using VarScan (Koboldt *et al.* 2009) with default settings. Both single nucleotide polymorphism (SNP) and indel variant positions were identified for each amplicon using minimum 'cut-off' frequencies between 0.01–0.2, in increments of 0.01. Variants identified at a minimum 'cut-off' frequency of 0.02 were entered into the NCBI dbSNP database (http://ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?type=contact&handle=CAW_ET). Specific SNPs can be identified within the reference sequences of each gene (*Ci*VDR/PXRα: KC561370, *Ci*VDR/PXRβ: KC561371 and *Bs*VDR/PXRα: KC561372) by string searches using 25 nucleotides 5' and 3' to each SNP listed within the dbSNP database.

Numbers of transitions, transversions and the transitional to transversional ratios (Ti/Tv) per site were calculated using the Tamura-Nei model as implemented in the MEGA 5 software package (Tamura & Nei 1993). Three population genetic parameters (Watterson's θ, Tajima's D (D_T) and π) were estimated using the sliding window approach implemented in the PoPoolation software (Kofler *et al.* 2011). Since D_T is sensitive to variation in coverage, in part due to sequencing errors, D_T was estimated by subsampling all reads to coverage of 1000 bases using a minimum count of two and a minimum quality of 20. This analysis only allows for relative comparison among the study populations and not for direct comparisons with other studies because D_T depends on coverage and window size. Values for all population statistics were calculated in non-overlapping 50 bp windows across each gene.

Non-synonymous and synonymous SNPs identified at a minimum variant frequency of 0.02 were mapped onto each of the three reference sequences (KC561370–72). Regional pairwise calculations (reference sequence vs. variant sequence) were used to calculate d_N/d_S ratios in a sliding window as implemented in JCoDA (Steinway *et al.* 2010). Using the Yang and Nielsen (2000) substitution model, d_N/d_S ratios were calculated with a window size of 200 codons and an increment jump of 25 codons.

2.3 Results

2.3.1 Determination of tunicate VDR/PXRα and β partial coding sequences

Partial coding sequences from the two *C. intestinalis* VDR/PXR orthologues (*Ci*VDR/PXRα and *Ci*VDR/PXRβ) and the *B. schlosseri* VDR/PXRα orthologue (*Bs*VDR/PXRα) were amplified from appropriate cDNA templates (*C. intestinalis*: gut cDNA; *B. schlosseri*: total colony cDNA) using the primer pairs listed in Appendix 1.

The amplified sequences were confirmed as genuine VDR/PXR orthologues using: (i) the Basic Local Alignment Search Tool (BLAST) to find annotated GenBank entries and; (ii) phylogenetic analyses using alignments of the corresponding predicted protein sequences. In nucleotide BLAST searches (BLASTN) of GenBank (nucleotide database, no taxonomic restrictions) the *Ci*VDR/PXRα sequence (KC561370) displayed 99% identity (E value = 0) with coordinates 431–1756 of the previously reported *Ci*VDR/PXRα sequence (NM_001078379). This sequence had been derived from the *C. intestinalis* genome sequence, in combination with strong EST support (AB210742; NCBI Gene ID: 778791; Ensembl transcript: ENSCINT00000031407). The BLASTN searches of GenBank (nucleotide database) using the *Ci*VDR/PXRβ sequence (KC561371) as the query revealed high sequence identity with sections of a previously reported *C. intestinalis* VDR/PXRβ sequence (NM_001044366; NCBI Gene ID: 751590; Ensembl transcript: ENSCINT00000030933) derived from the *C. intestinalis* genome sequence in combination with some EST support. Examination of the KC561371/NM_001044366 alignments revealed regions of near identity interspersed with non-aligned regions. The most parsimonious interpretation of these alignments is that KC561371 and NM_001044366 are derived from splice variant transcripts from the same locus.

Both BLASTN and translated nucleotide BLAST (TBLASTN) searches of GenBank (nucleotide database, no taxonomic restrictions) using the putative *Bs*VDR/PXRα partial coding sequence (KC561372) identified *Ci*VDR/PXRα (NM_001078379) as the most homologous sequence on the GenBank nucleotide database (BLASTN: identity = 70% over coordinates 1214–1477 of NM_001078379, E value = 9e-25; TBLASTN: identity = 52%, E value = 3e-94). Additionally, BLASTN searches of a *B. schlosseri* EST database (http://octopus.obs-vlfr.fr/public/botryllus/blast_botryllus.php) provided EST support for the *Bs*VDR/PXRα (KC561372) sequence (BLASTN: identity = 98% over coordinates 783–1788 of Bot_oas_2431, E value = 0.0).

The orthologous relationships of the three tunicate amplified cDNA sequences were also examined by phylogenetic analysis based on alignments of conserved sections of protein sequences. Predicted protein sequences were derived from KC561370 (421 residues), KC561371 (480 residues) and

KC561372 (333 residues). The putative DBD and LBD domains were predicted using three different methods (Figure 2.1, Appendix 2). The derived phylogeny placed the three tunicate sequences in the same NR clade as the vertebrate VDR (NR1I1), PXR (NR1I2) and CAR (NR1I3) genes supporting their designation as VDR/PXR orthologues (Figure 2.2).

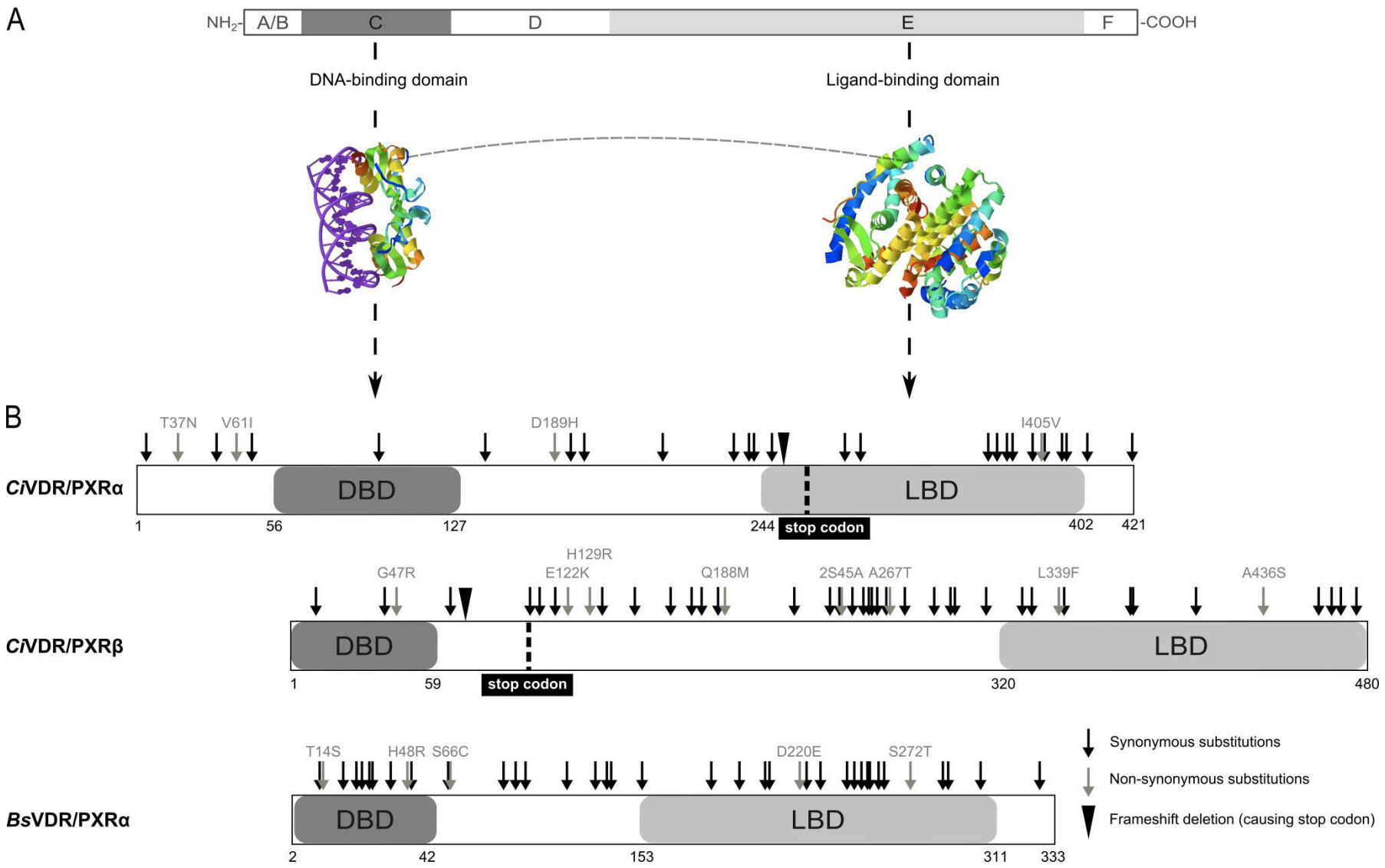

Figure 2.1 Genetic diversity within the primary structures of the predicted tunicate xenobiotic receptor proteins.

Figure 2.1 (cont.) (**A**) Schematic diagram of the generic nuclear receptor structure with conserved domains indicated: N-terminal A/B domain (A/B), DNA-binding domain (DBD; C), flexible hinge region (D), ligand-binding domain (LBD; E) and C-terminal activation domain (F). (**B**) Schematic primary structures of the predicted *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα), *C. intestinalis* VDR/PXRβ and *Botryllus schlosseri* VDR/PXRα proteins with residue/codon numbers following the corresponding GenBank entries: *Ci*VDR/PXRα: KC561370; *Ci*VDR/PXRβ: KC561371; *Bs*VDR/PXRα: KC561372. The DBDs (dark grey) and LBDs (light grey) predicted by the Simple Modular Architecture Research Tool (SMART; Letunic *et al*. 2012) are highlighted. Approximate locations of single nucleotide polymorphisms (SNPs) in the corresponding nucleotide sequences are indicated by arrows (synonymous: black; non-synonymous: grey). Alternative residues associated with non-synonymous SNPs are indicated above the grey arrows. The approximate locations of one base pair indel variants (black triangle) are indicated along with the position of the first stop codon generated 3' to the indel-associated frameshift (dashed line).

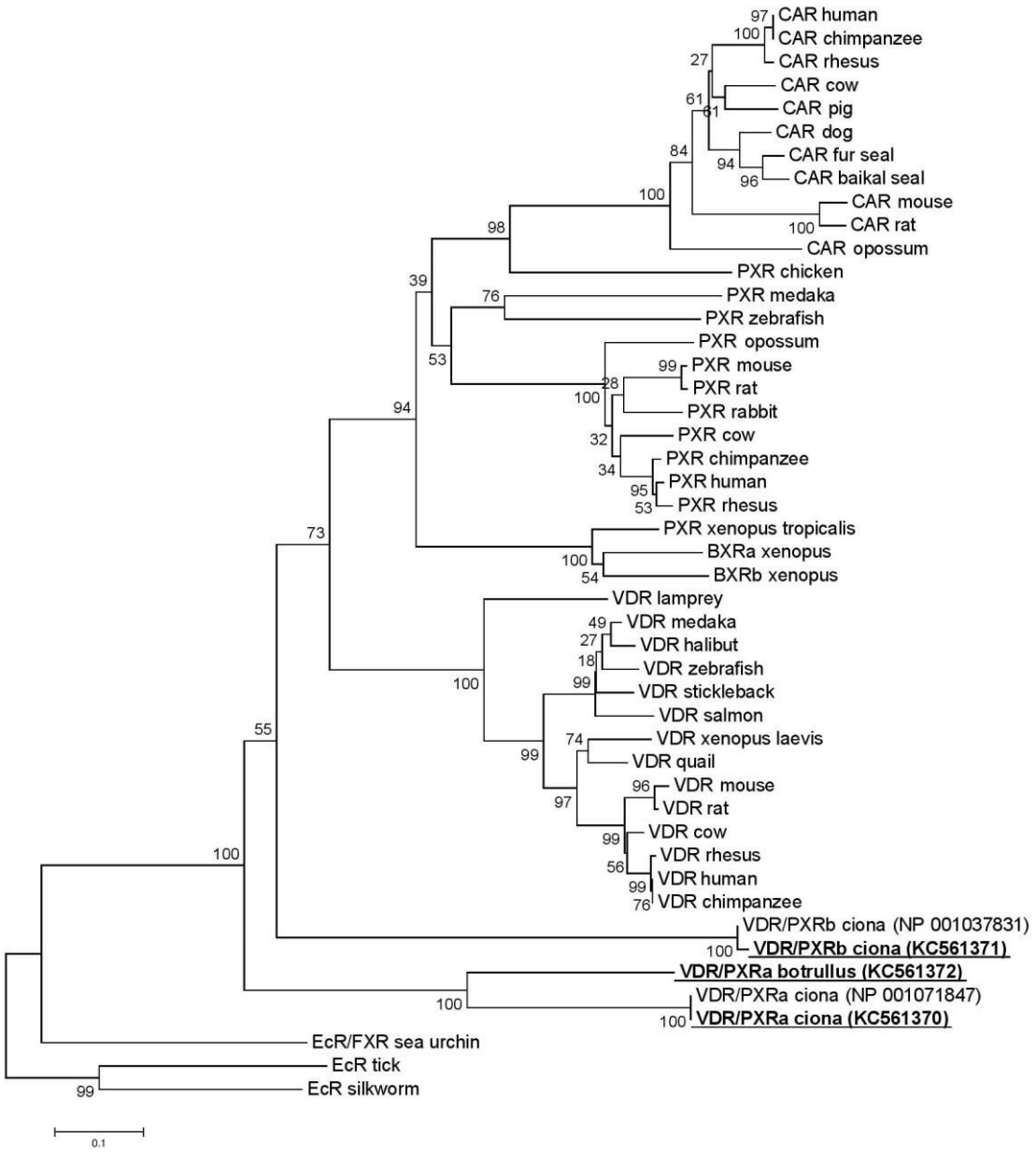

Figure 2.2 Molecular phylogeny of nuclear receptors including the three tunicate xenobiotic receptors investigated in this study.

Predicted DNA-binding domain (DBD) and ligand-binding domain (LBD) sequences from a range of metazoan nuclear receptors (NRs) were aligned and used to generate neighbour-joining trees. Bootstrap values were calculated from 1000 replicates. The sequence alignment used, along with corresponding GenBank accession numbers, is shown in Appendix 3. The three tunicate NR sequences generated in this study are indicated in underlined bold text.

2.3.2 Detection of VDR/PXRα and β transcripts in the *Ciona intestinalis* digestive tract

Both conventional PCR and quantitative PCR (qPCR) were used to detect *Ci*VDR/PXRα and β transcripts in five sections of the *C. intestinalis* gut (Figure 2.3). A *Ciona intestinalis* cytoskeletal actin gene orthologue (GenBank acc. no.: AJ297725) was used as an internal control for both RNA integrity and cDNA synthesis. For all three *C. intestinalis* transcripts investigated, PCR primer pairs were designed to flank at least one intron sequence to ensure that amplicons generated from any contaminating genomic DNA (gDNA) could be distinguished from those amplified from cDNA, on the basis of their differing lengths (Appendix 1B). Using conventional PCR and amplicon visualisation on agarose gels, *Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Ci*actin transcripts were detected in the five regions of the digestive tract with no indication of gDNA contamination as indicated by bands of the expected sizes on agarose gels (Appendix 4). Given the non-quantitative nature of conventional PCR, qPCR was used to determine transcript levels in total RNA extracted from five digestive tract regions. The estimated concentrations of the three transcripts (*Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Ci*actin) were calculated as molecules/ng cDNA, based on plasmid standards, and grouped into five concentration ranges (Figure 2.3). Although transcripts for all three genes were detected in all five sections of the *C. intestinalis* gut, the lower part of the digestive tract appeared to have higher transcript levels for all three genes ($p < 0.01$, Figure 2.3, Appendix 5).

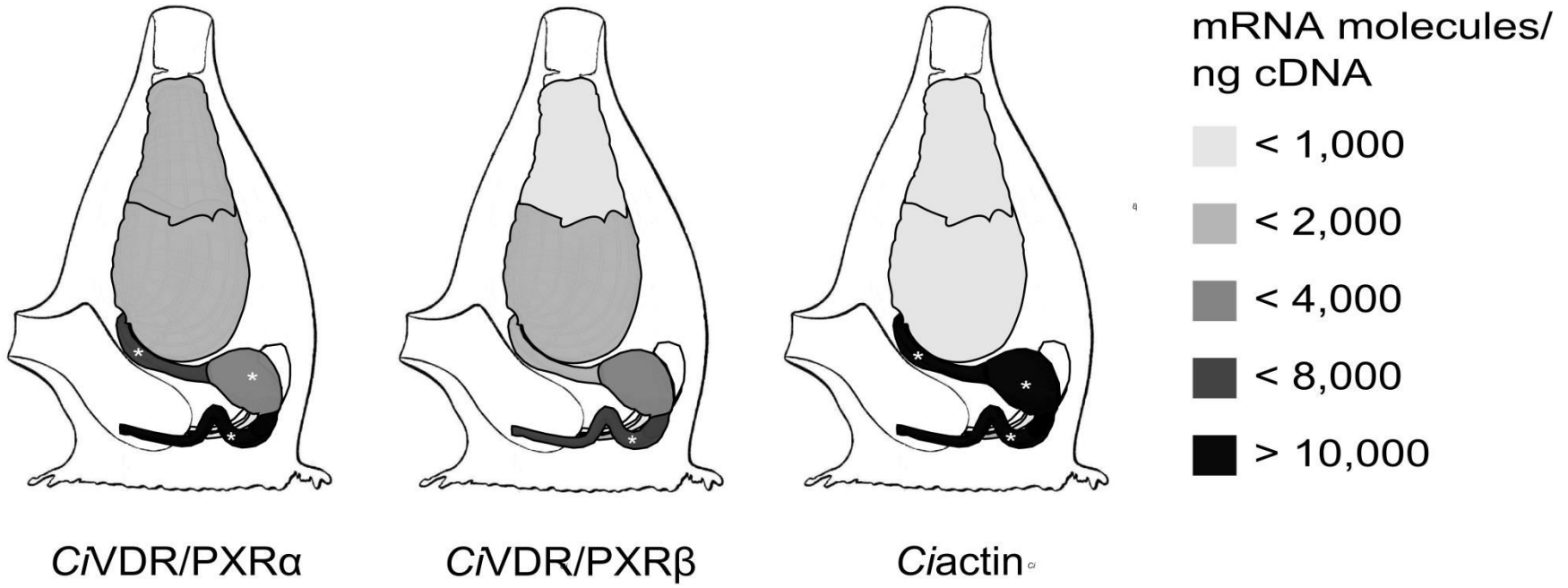

Figure 2.3 Schematic summary of gene transcripts in gut regions of *Ciona intestinalis* including two xenobiotic receptors investigated in this study.

Schematic summary of *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ) and *C. intestinalis* actin (*Ci*actin) transcript levels in five gut regions as determined using quantitative polymerase chain reaction. Estimated numbers of mRNA molecules/ng cDNA were averaged from triplicate amplification reactions. Significant differences ($p < 0.01$) in gene expression between tissues are indicated (*).

2.3.3 Sequence polymorphisms detected in tunicate VDR/PXR amplicons

Allelic variation in the three tunicate VDR/PXR coding sequences was assessed by sequencing pooled amplicons derived from 30 individual *C. intestinalis* animals (*Ci*VDR/PXRα and *Ci*VDR/PXRβ) and 30 *B. schlosseri* colonies (*Bs*VDR/PXRα) using the Illumina™ MiSeq System. After filtering and quality control, the paired-end sequences were mapped onto the three reference sequences (Appendix 6). Sequence coverage at each reference sequence nucleotide position was calculated up to a maximum coverage of 40,000 fold. The lowest coverage was 19,374 fold at position 127 of *Ci*VDR/PXRα (KC561370) and the average coverage was 39,454 fold (Appendix 7).

The sequence data were examined for sequence polymorphisms. Given the significant error rates associated with the Illumina™ MiSeq System (Harismendy *et al.* 2009; Nakamura *et al.* 2011), consideration was given to the minimum variant frequency necessary for a polymorphism to be a genuine polymorphism rather than a technical artefact. As expected, the number of polymorphisms predicted in all three amplicon sequence pools increased as the minimum frequency for acceptance was reduced from 0.2 to 0.01 in 0.01 decrements (Appendix 8). As the *C. intestinalis* derived amplicon pools were generated from 30 diploid animals, the minimum expected frequency for a genuine polymorphism would be 1/60 (ca. 1.7%). Experimental errors inherent in the quantification and pooling of amplicons might result in a genuine variant being present in the data-set at a frequency <1.7%. Based on these assumptions, a value of 0.02 (2%) was selected as the minimum frequency for polymorphisms in the amplicon data-set to be considered genuine (Appendix 8). Although applying a 'cut off' value of 0.02 carries the risk of excluding genuine variation, such exclusion was considered preferable to inclusion of false variants which may have occurred if the minimum variant frequency was set lower. The coordinates of the SNPs and indels identified are shown schematically in Figure 2.1, and can be obtained from Appendix 8 and from the GenBank dbSNP entries (Section 2.2.7).

The estimated SNP numbers were as follows: *Ci*VDR/PXRα (KC561370, 1326 bp): 28 SNPs, 2.11 SNPs/100 bp; *Ci*VDR/PXRβ (KC561371, 1569 bp): 44 SNPs, 2.8 SNPs/100 bp and *Bs*VDR/PXRα (KC561372, 1006 bp): 39 SNPs, 3.88 SNPs/100 bp (Table 2.2). Most SNPs were transitions (Ti): *Ci*VDR/PXRα: 78%, *Ci*VDR/PXRβ: 71%, *Bs*VDR/PXRα: 80%, with transition/transversion rate ratios (Ti/Tv) of 3.7 (*Ci*VDR/PXRα), 2.4 (*Ci*VDR/PXRβ) and 3.9 (*Bs*VDR/PXRα; Appendix 9). The majority of SNPs were synonymous: *Ci*VDR/PXRα: 86%, *Ci*VDR/PXRβ: 70% and *Bs*VDR/PXRα: 87% (Table 2.2, Figure 2.1). Mean nucleotide diversity (π) and expected population mutation rate (Watterson's Theta; θ_W) were estimated for each of the coding sequences using sliding window calculations as outlined in Section 2.2.7. Tajima's D (D_T) values were negative for all three coding sequences indicating purifying selection (Table 2.2). Regional pairwise calculations were performed via sliding window calculation of the ratio of non-synonymous substitutions per non-synonymous site (d_N) to synonymous substitutions per synonymous site (d_S) which were <0.2 across all regions of the three amplicons (Table 2.2).

Table 2.2 Summary statistics for polymorphisms detected in tunicate xenobiotic receptor orthologues.
Polymorphisms are listed for partial coding sequences of the *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα). Standard deviations (SD) are shown in brackets.

	Length (bp)	**T_{SNPs}**	**1 bp indel**	**f_{SNPs}**	**Syn_{SNPs}**	**$NonSyn_{SNPs}$**	**d_N/d_S**	**π (± SD)**	**θ_w (± SD)**	**D_T (± SD)**
*Ci*VDR/PXRα	1326	28	1	2.11	24 (86%)	4 (14%)	0.055	0.007 (0.006)	0.076 (0.015)	-3.481 (0.305)
*Ci*VDR/PXRβ	1569	44	1	3.19	21 (70%)	9 (30%)	0.083	0.009 (0.007)	0.075 (0.011)	-3.343 (0.362)
*Bs*VDR/PXRα	1006	39		3.87	34 (87%)	5 (13%)	0.105	0.013 (0.009)	0.081 (0.014)	-3.176 (0.456)

Abbreviations: SNP, single nucleotide polymorphism; T_{SNPs}, total SNP number; bp, base pair; f_{SNPs}, SNP frequency (SNP/100 bp); Syn_{SNPs}, synonymous SNPs; $NonSyn_{SNPs}$, non-synonymous SNPs; d_N/d_S, rate of non-synonymous substitutions per non-synonymous site (d_N) to synonymous substitutions per synonymous site (d_S) calculated using sliding window analysis (Steinway *et al.* 2010); π, mean pairwise nucleotide diversity (Nei & Gojobori 1986); θ_w, expected population mutation rate (Watterson 1975); D_T, Tajima's D (Tajima 1989).

2.3.4 Frameshift polymorphisms in the *Ciona intestinalis* VDR/PXR coding sequences

Single base indel polymorphisms were identified in both the *Ci*VDR/PXRα (+/- coordinate 834 of KC561370) and *Ci*VDR/PXRβ (+/- coordinate 235 of KC561371) coding sequences (Figure 2.1, Table 2.2). The frequencies of these variants within the pooled amplicon sequence dataset were 8.4% and 2.1%, respectively. Alleles carrying these one base pair frameshifts were predicted to encode VDR/PXR proteins that consist of a DBD but lacking a LBD (Figure 2.1). In support of the reliability of these indel polymorphisms, the *Ci*VDR/PXRα one base pair indel was also found in parallel investigations in which amplicons were first cloned into pGemT-easy vectors and then sequenced using Sanger sequencing (data not shown). No indels were detected in the *Bs*VDR/PXRα amplicon pool when applying the minimum variant frequency 'cut off' value of 0.02 (Figure 2.1, Table 2.2, Appendix 8). However, at a 'cut off' minimum variant frequency of 0.01 additional indels were found in all three tunicate VDR/PXR coding sequences and it is possible that some of these are genuine (Appendix 8).

2.3.5 Haplotype diversity in two *Botryllus schlosseri* populations from different geographical locations

Haplotype diversity of the *B. schlosseri* VDR/PXRα partial coding sequence was compared between populations in New Zealand (Nelson Marina) and on the eastern Mediterranean coast (Michmoret Beach, Israel). To allow complete VDR/PXRα haplotype sequences to be identified with confidence, sequences were generated from cloned amplicons using Sanger sequencing. *Botryllus schlosseri* VDR/PXRα amplicons were amplified from cDNA templates from ten randomly selected colonies from both geographical locations and ligated into a T-tailed cloning vector before transformation into *E .coli*. Plasmids were purified from three independent *E. coli* colonies corresponding to each ligation/amplicon and their *B. schlosseri* VDR/PXRα inserts sequenced.

Fifteen distinct haplotypes were found amongst the 30 sequences sampled from the Mediterranean population with nine haplotypes found in the 30 sequences from the New Zealand population. Consistent with this result, the SNP frequency was higher in the Mediterranean population (42 SNPs/1006 bp; 4.17 SNPs/100 bp) than in the New Zealand population (33 SNPs/1006 bp; 3.28 SNPs/100 bp, Appendix 10) with this difference being statistically significant (two-sample Kolmogorov-Smirnov test, $p = 0.01$). In the Mediterranean sample, 24% (10/42) of the SNPs were non-synonymous with 11% (4/33) non-synonymous in the New Zealand sample (Appendix 10). Most of the SNPs (85%) found in New Zealand were also present in the Mediterranean population, while 69% of SNPs found in the Mediterranean were also present in the New Zealand population (Appendix 10). Despite the shared SNPs, no complete haplotypes were shared between the Mediterranean and New Zealand populations.

2.4 Discussion

2.4.1 Determination of tunicate VDR/PXRα and β sequences

This chapter confirms the majority of the *C. intestinalis* VDR/PXRα coding sequence previously inferred from combined analyses of *C. intestinalis* genomic and EST sequence data (Dehal *et al.* 2002; Ekins *et al.* 2008). In contrast, the amplified *C. intestinalis* VDR/PXRβ sequences had apparent insertions of 21 and 106 codons when aligned with the *C. intestinalis* VDR/PXRβ sequence (NM_001044366) that was inferred from genomic and EST sequences. Given the limited EST support for the NM_001044366 sequence, its accuracy must be regarded with caution. The most conservative approach is to regard the *Ci*VDR/PXRβ sequence amplified in this chapter (KC561371) and the NM_001044366 as splice variants. The closest homologue of *Bs*VDR/PXRα was *Ci*VDR/PXRα, suggesting that the VDR/PXRα orthologue is also present in *B. schlosseri*. This is supported by the recent release of the *B. schlosseri*

genome (Voskoboynik *et al.* 2013), which contains two genomic contigs encoding VDR/PXRα.

Protein sequences predicted from the three tunicate sequences contained distinct DBDs and LBDs (Figure 2.1), thus conforming to the generic NR structure (Marchler-Bauer *et al.* 2011; Letunic *et al.* 2012; Punta *et al.* 2012). Although all three protein domain prediction programmes returned consistent results, these *in silico* predictions are based on databases consisting of proteins with known structural domains/sequences (Schultz *et al.* 1998; Lubec *et al.* 2005; Letunic *et al.* 2012). In the case of NRs, databases are largely composed of typical NRs (e.g. steroid hormone receptors), which have highly selective LBDs that bind a structurally limited range of ligands (Zhang *et al.* 2004; Krasowski *et al.* 2005b). As the LBD of PXR orthologues is expected to be both larger and more flexible than is typical of NR LBDs, the predicted tunicate VDR/PXR LBDs need to be treated with caution (Watkins *et al.* 2001; Watkins *et al.* 2003; Chrencik *et al.* 2005). To better assess the reliability of the VDR/PXR LBD software predictions, the predicted human PXR LBD was compared with the LBD coordinates that had been determined experimentally. Watkins *et al.* (2001) concluded from empirical crystallography data that the human PXR LBD was formed by residues 142–431. In contrast, the human PXR (NM 003889) LBD domain was considerably smaller according to software-based predictions by SMART (residues 245–404), NCBI CD (residues 236–428) and Pfam (residues 223–426). All three programmes underestimated the size of the human PXR LBD, most noticeably at the N-terminal boundary. Applying an N-terminal extension of approximately 100 residues to the SMART predicted *Ci*VDR/PXRα and *Bs*VDR/PXRα LBD would place the N-terminal boundaries of these LBDs close to their respective DBDs. In contrast, there are approximately 250 residues between the SMART predicted *Ci*VDR/PXRβ DBD C-terminus and LBD N-terminus. In the absence of functional and/or structural information it is not possible to determine if the *Ci*VDR/PXRβ protein has a larger LBD than its VDR/PXRα paralogue. Thus, *Ci*VDR/PXRβ may be able to bind larger ligands or may simply have an extended linker region separating the DBD and LBD.

2.4.2 Expression of VDR/PXRα and β transcripts in the *Ciona intestinalis* digestive tract

Ciona intestinalis VDR/PXRα and VDR/PXRβ transcripts were detected throughout the digestive tract. These data suggested that VDR/PXRα expression levels may be higher in the lower part of the digestive tract. However, transcript levels of the internal control (actin) were also elevated making interpretation difficult. Nonetheless, detection of VDR/PXRα and VDR/PXRβ mRNA in all parts of the *C. intestinalis* digestive tract is consistent with functional roles as xenobiotic receptors— by analogy with the vertebrate PXR which is predominantly expressed in liver and intestine, which are both sites for xenobiotic metabolism (Xie *et al.* 2000; Staudinger *et al.* 2001).

2.4.3 Variation in the tunicate VDR/PXRα and β sequences

The SNP frequencies found in the three tunicate VDR/PXR coding regions (approximately 3 SNPs/100 bp) are comparable to rates reported from tunicate genome sequencing projects: *B. schlosseri*, *C. intestinalis*, *Ciona savignyi* and *Oikopleura dioica* (Dehal *et al.* 2002; Small *et al.* 2007; Caputi *et al.* 2008; Denoeud *et al.* 2010; Satou *et al.* 2012; Tsagkogeorga *et al.* 2012; Voskoboynik *et al.* 2013). Tunicates are known to have a high level of genetic diversity, which can be up to ten times higher than in human (1 SNP/1000 bp), zebrafish and fugu (4 SNPs/1000 bp; Wang *et al*. 1998; Aparicio *et al*. 2002; Guryev *et al*. 2006; Wheeler *et al*. 2008). These high levels of genetic diversity are consistent with a large effective population size and a high per-year mutation rate (μ), which is two to six times higher in tunicates than in vertebrates due to a short generation time (Lambert 2005; Thomas *et al.* 2010; Tsagkogeorga *et al.* 2012).

Among all identified polymorphisms, transitions (Ti) outnumbered transversions (Tv) by about three to one in partial coding regions of *Ci*VDR/PXRα, *Ci*VDR/PXRβ and *Bs*VDR/PXRα. The average transition/transversion ratios (Ti/Tv = 3.3) in the three coding sequences were similar to Ti/Tv ratios reported

for *C. savignyi* (Small *et al.* 2007). This contrasts with Ti/Tv ratios in mammalian coding sequences (Ti/Tv = 2), which can be attributed to larger biases against CpG sequences in mammalian coding sequences than in tunicate coding sequences (Moriyama & Powell 1996; Cargill *et al.* 1999; Lindblad-Toh *et al.* 2000).

Although high SNP frequencies were found, the great majority of SNPs in all three tunicate VDR/PXR coding sequences were synonymous. Together with the negative Tajima's D values (D_T <-3) and low d_N/d_S ratios (<0.2), the sequence variation data indicates that tunicate VDR/PXR coding sequences are under strong purifying selection. These findings are consistent with recent population genomics analyses of *C. intestinalis* which reported a very low average d_N/d_S ratio (0.07) in combination with high SNP frequencies (approximately 5.7 SNPs/100 bp; Tsagkogeorga *et al.* 2012). This is consistent with tunicates having elevated mutation rates and large effective population sizes in combination with strong purifying selection (Kimura 1983; Lynch 2008; Tsagkogeorga *et al.* 2012; Berná & Alvarez-Valin 2014).

Overall, this interpretation would be the most parsimonious explanation of the SNP types, and frequencies, detected in the tunicate VDR/PXR coding sequences. However, single amino acid changes can have significant functional consequences. Non-synonymous SNPs in the human PXR gene alter PXR protein function as measured by basal and/or induced transactivation of CYP3A promoters (Hustert *et al.* 2001). Similarly, two natural human CAR sequence variants were shown to alter the receptor's constitutive (ligand-independent) transactivation activity (Ikeda *et al.* 2005). The effects of single residue differences can be extended to inter-taxa difference. For example, differences in human and mouse PXR activation by rifampicin can be attributed to differences at a single position: human PXR Leu_{308}/rat PXR Phe_{305} (Tirona *et al.* 2004).

2.4.4 Frameshift polymorphisms

Both *C. intestinalis* VDR/PXR genes had at least one allele containing a single-base deletion resulting in the predicted proteins having a DBD but lacking a

LBD. The persistence of such frameshift alleles in natural populations, at low but significant frequencies, suggests that they are maintained by some form of balancing selection. The corresponding predicted proteins that lack LBDs seem likely to mediate constitutive, rather than ligand-activated, gene transcription. In support of this interpretation, NR variants lacking a LBD have been shown to constitutively activate transcription in vertebrates (e.g. human androgen receptor; Tepper *et al*. 2002; Marcias *et al*. 2010) and insects (e.g. Knirps; Nauber *et al*. 1988). Similarly, a number of NR splice variants have been described that translate into proteins lacking LBDs and which constitutively activate target genes (e.g. human estrogen receptor; Chaidarun *et al*. 1998). To date, no equivalent splice variants lacking a LBD have been described for vertebrate PXRs (Hustert *et al.* 2001). In short, the VDR/PXRα and β frameshift alleles are likely to activate target gene transcription constitutively which may confer advantages over ligand-activated transcription in some contexts.

2.4.5 Haplotype diversity in two *Botryllus schlosseri* populations from different geographical locations

The *B. schlosseri* population from the eastern Mediterranean coast displayed greater VDR/PXRα genetic diversity than the New Zealand population. The lower genetic diversity of New Zealand populations, when compared to Mediterranean populations, has been noted in previous studies using microsatellite loci (Ben-Shlomo *et al.* 2001; Paz *et al.* 2003). This observation has been intrepreted as reflecting founder effects associated with introduction events to New Zealand (Ben-Shlomo *et al.* 2001). Consistent with this interpretation, the majority of VDR/PXRα SNPs found in the New Zealand population (>85%) were present in the Mediterranean population, whereas 30% of the SNPs in the Mediterranean population were unique to that population. No complete haplotype sequences were shared between the two populations. This is consistent with the high rates of genetic variation and recombination frequencies characteristic of tunicate genomes (Simmen & Bird 2000; Satoh & Levine 2005; Caputi *et al.* 2008; Satou *et al.* 2012; Tsagkogeorga *et al.* 2012).

2.5 Conclusion

The mRNA sequences of two *C. intestinalis* and one *B. schlosseri* VDR/PXR orthologues have been characterised. All three corresponding proteins were predicted to have the typical NR structure, containing distinct LBDs and DBDs. The *Ci*VDR/PXRβ LBD may be larger than the *Ci*VDR/PXRα LBD, suggesting that it might accommodate larger ligands. All three transcripts harboured high rates of sequence polymorphism with evidence of purifying selection. Examination of d_N/d_S ratios over an entire gene is extremely conservative and is unlikely to detect positive selection acting on a few sites (Jensen *et al.* 2007). It is also possible for loci to be under selection without yielding statistically significant results in tests for selection (Przeworski *et al.* 2005; Teshima *et al.* 2006). However, allelic variants that contained one base pair frameshift polymorphisms were detected in both *C. intestinalis* VDR/PXR orthologues. The resulting proteins were predicted to contain a DBD but lacking a LBD. Such VDR/PXR variants may mediate constitutive transcription of target genes. Haplotype variation of *B. schlosseri* VDR/PXRα was found to be higher in a population from the Mediterranean than New Zealand, which is consistent with founder effects during colonisation. The data presented in this chapter provide a foundation for further investigations into the molecular evolution, population genetics and function of tunicate NRs thought to be involved in the detection of marine bioactive compounds.

2.6 References

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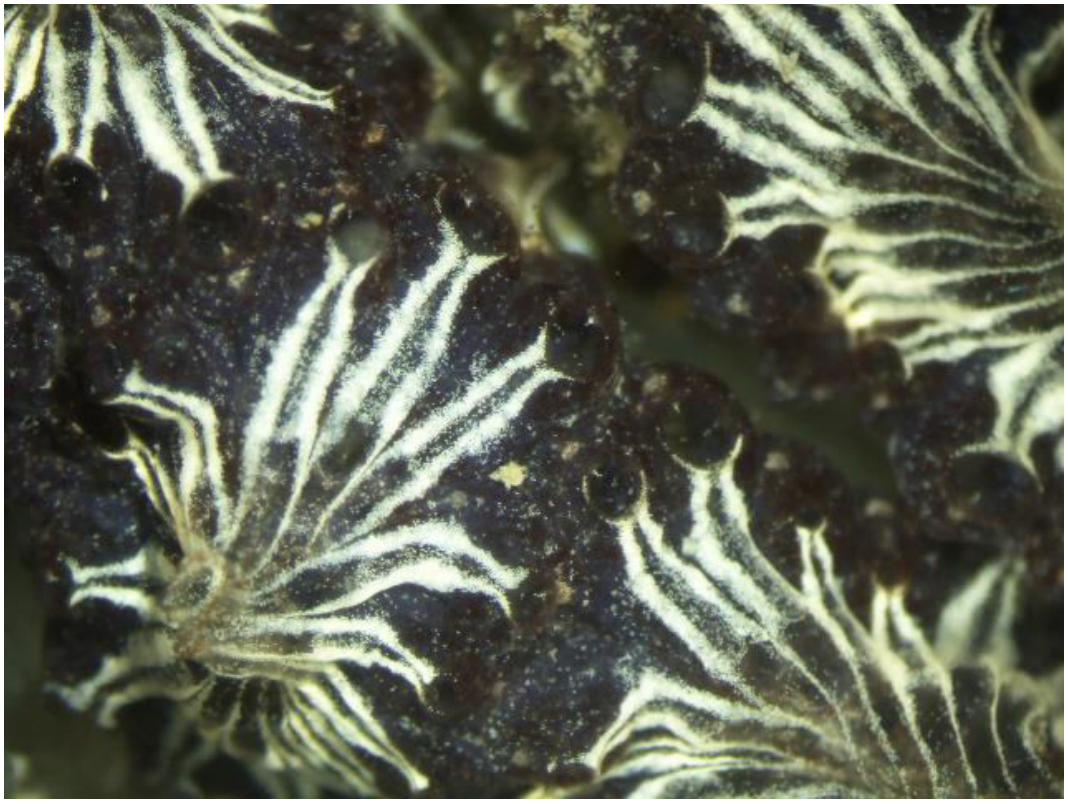

Botryllus schlosseri colonies from Nelson marina, New Zealand. Photograph provided by Prof Baruch Rinkevich, National Institute of Oceanography, Israel.

Part Two

Functional characterisation of tunicate xenobiotic receptors and their application in yeast-based bioassays

CHAPTER THREE

Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast

3.1 Introduction

Marine microalgal taxa can produce highly toxic chemicals that can cause widespread poisoning within marine ecosystems. These toxic compounds may also affect human health, particularly when accumulated in filter-feeding shellfish (Wear & Gardner 2001; Wang 2008; MacKenzie *et al.* 2011; Zhang *et al.* 2013). Over the past two decades, internationally accepted methodologies for the detection of such natural marine biotoxins have become increasingly based on chemical, rather than biological, assays (Gerssen *et al.* 2010; Suzuki & Quilliam 2011; McNabb *et al.* 2012). While such chemistry-based detection methods are generally reliable and highly specific, they require specialised equipment and technically skilled personnel. Detailed structural knowledge regarding the targeted biotoxins is also essential, thus unknown marine biotoxins cannot be detected (Humpage *et al.* 2010; Nicolas *et al.* 2014). Although a range of *in vitro* bioassay tests for microalgal biotoxins has been developed (Bovee *et al.* 2011; Banerjee *et al.* 2013; Nicolas *et al.* 2014), the most widely-used bioassay remains the unreliable and ethically questionable mouse mortality bioassay (Botana *et al.* 2009; Buckland 2010; Stewart & McLeod 2014). There is, therefore, a need for new, ethically-acceptable, simple, robust and inexpensive bioassays (Vilarino *et al.* 2010; Hashimoto *et al.* 2011; Nicolas *et al.* 2014).

In Chapter Two, putative xenobiotic receptors that are orthologues to vertebrate pregnane X receptor (PXR) and vitamin D receptor (VDR) were successfully identified in two tunicate species, *Ciona intestinalis* and *Botryllus schlosseri* (abbreviated VDR/PXR). Genomic sequence-based analyses are always

tentative and functional data are needed for the confident assignment of these proteins as a functional xenobiotic receptor. The *C. intestinalis* VDR/PXRα (*Ci*VDR/PXRα) has been characterised in terms of its ligand-binding domain (LBD) mediating ligand-dependent luciferase reporter gene expression when fused to the generic GAL4 DNA-binding domain (GAL4-DBD) and expressed in a mammalian cell line (Ekins *et al.* 2008). Using this bioassay, three synthetic chemicals and two microalgal biotoxins (pectenotoxin-2 (PTX-2) and okadaic acid) were identified as *Ci*VDR/PXRα agonists and a common ligand pharmacophore was defined (Reschly *et al.* 2007; Ekins *et al.* 2008; Fidler *et al.* 2012). Collectively, these studies established that the *Ci*VDR/PXRα receptor has ligand-binding characteristics consistent with a natural role in detecting marine xenobiotics, including microalgal biotoxins. This makes it a potential sensor element for use in bioassays (Fidler *et al.* 2012).

In this chapter, I report the development of recombinant yeast (*Saccharomyces cerevisiae*) strains that express the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD in combination with the GAL4-DBD and a generic transcription activation domain (VP16-AD). These recombinant proteins mediate ligand-dependent expression of the easily assayed *lacZ* reporter gene to facilitate detection of probable ligands for tunicate VDR/PXRα receptors. The bioassay strains were tested with four synthetic toxicants and five natural toxins including three microalgal biotoxins (pectenotoxin-11 (PTX-11), okadaic acid and portimine) and two freshwater cyanobacterial toxins (anatoxin-A and microcystin-RR).

3.2 Materials and methods

3.2.1 Compounds tested in the yeast bioassay

Four synthetic compounds (*n*-butyl-*p*-aminobenzoate, carbamazepine, *p*-aminobenzoic acid and bisphenol-A) tested in the yeast bioassay were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.; Table 3.1). The algal toxin okadaic acid was purchased from Sapphire Bioscience (Waterloo, Australia), microcystin-RR from DHI Lab Products (Horsholm, Denmark) and anatoxin-A was purchased from the National Research Council Canada (Ottawa, Canada). Pectenotoxin-11 (MacKenzie *et al*. 2013) and portimine (Selwood *et al.* 2013) were sourced from within the Cawthron Institute (Nelson, New Zealand; Table 3.2). Chemical structures are given in Figure 3.1. All compounds were dissolved in analytical grade ethanol (Merck, Whitehouse Station, NJ, U.S.A.) to form stock solutions. Serial dilutions were added to the yeast bioassay media at a final ethanol concentration of 1% (v/v; Bovee *et al*. 2004). Solubility of test compounds in ethanol was confirmed using the PubChem database (http://pubchem.ncbi.nlm.nih.gov/) and ethanol was chosen as the preferred standard solvent for consistency across all assays. In some cases, due to the limited solubility of some compounds, chemicals were also dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and tested in the yeast bioassays (Appendix 11).

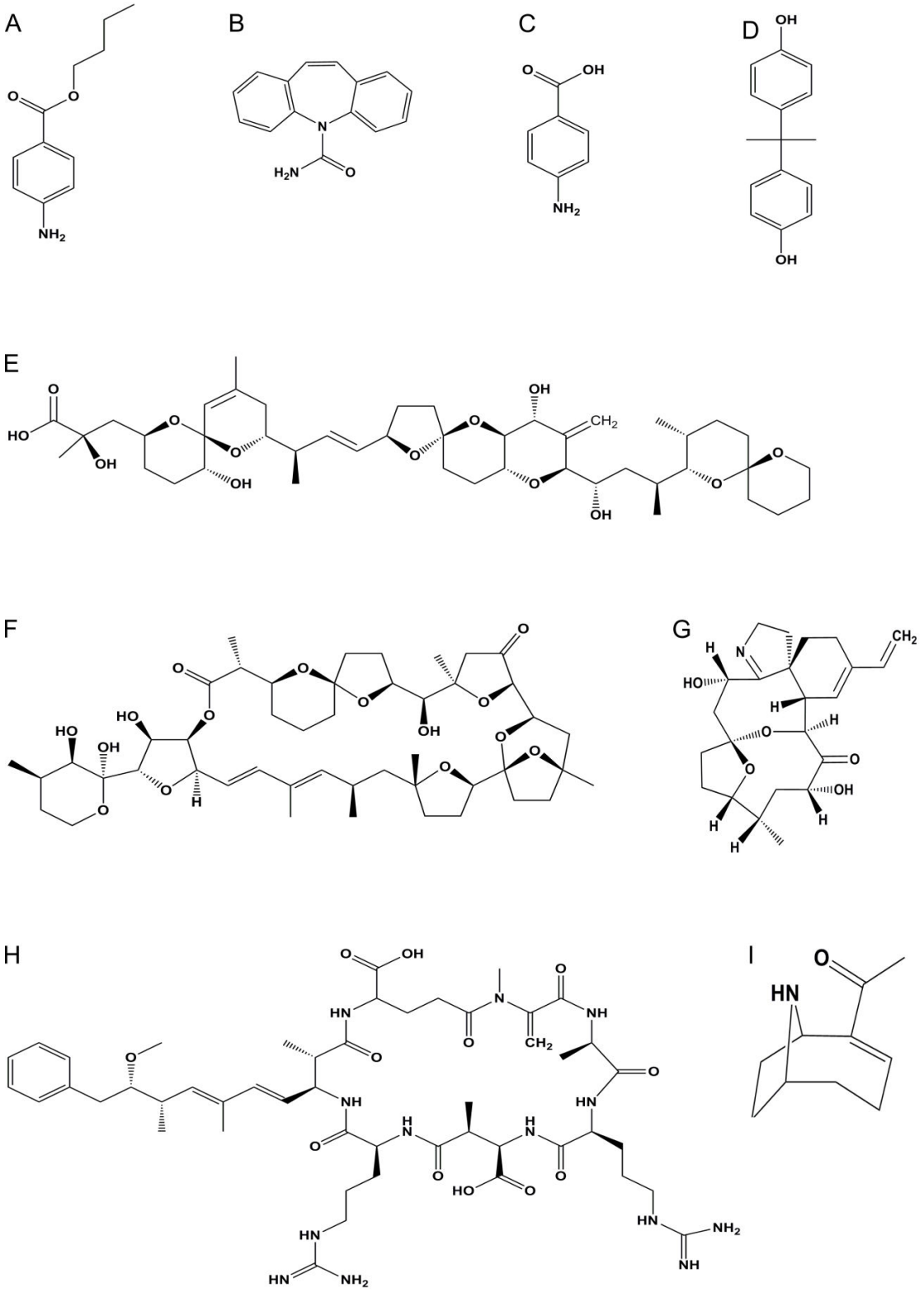

Figure 3.1 Structures of four synthetic chemicals and five algal toxins.

The following chemicals were tested in tunicate VDR/PXRα LBD-based yeast bioassays: (**A**) *n*-butyl-*p*-amino-benzoate, (**B**) carbamazepine, (**C**) *p*-aminobenzoic acid, (**D**) bisphenol-A, (**E**) okadaic acid, (**F**) pectenotoxin-11, (**G**) portimine, (**H**) microcystin-RR and (**I**) anatoxin-A.

3.2.2 Media

All media ingredients were purchased from Sigma-Aldrich unless stated otherwise. Minimal media (MM) was prepared following Routledge and Sumpter (1996) and Beresford *et al.* (2000). However, three amino acids (L-histidine, L-tyrosine and L-phenylalanine) were omitted. Minimal media (100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$ (Merck), 75 mM KOH, 1.7 mM $MgSO_4$, 0.38 mM L-leucine, 0.11 mM L-arginine, 0.13 mM L-methionine, 0.23 mM L-isoleucine, 0.16 mM L-lysine, 0.68 mM L-glutamic acid, 1.3 mM L-valine, 3.6 mM L-serine and 0.37 mM adenine) had its pH adjusted to 7.1 using 5 M KOH before $Fe_2(SO_4)_3$ solution was added to a final concentration of 2 µM. Minimal media was autoclaved (121°C, 15 min) and stored at room temperature. Growth media (GM) was prepared by supplementing MM as follows: 2% (w/v) D(+) glucose, 0.89 mM adenine, 0.17 mM uracil, 1.52 mM L-threonine, 0.71 mM L-aspartic acid, 1/200 volume of 200x vitamin stock solution (1.7 µM pantothenic acid, 11 µM myo-inositol, 1.9 µM pyridoxine HCl and 1.2 µM thiamine HCl, 0.082 µM biotin, filter-sterilised) and 1/1000 volume of 1000x trace elements solution (8.08 µM H_3BO_3 (Merck), 0.25 µM $CuSO_4.5H_2O$ (Merck), 0.6 µM KI, 2.64 µM $MnO_4S.4H_2O$ and 0.97 µM $Na_2MoO_4.2H_2O$, 2.47 µM $ZnSO_4.7H_2O$). For corresponding solid media, agar was added at 2% (w/v).

3.2.3 Generation of plasmid constructs

All the plasmid constructs used in this study were derived from the 7.3 kilo base (kb) yeast expression vector pGBKT7 (Catalogue no. 630443, Clontech Laboratories Inc., Mountain View, CA, U.S.A.). The pGBKT7 vector encodes 147 residues of the GAL4-DBD located 5' to an adjacent multiple cloning site (MCS; Figure 3.2). Transcription of GAL4-DBD fusion genes is initiated from a constitutive *alcohol dehydrogenase 1* (*ADH1*) promoter (P_{ADH1}) and transcription is terminated by an *ADH1* transcription termination signal (T_{ADH1}). Selection for the pGBKT7 plasmid is mediated by kanamycin resistance (Kan^R) in *Escherichia coli* and by a nutritional marker gene (*TRP1*) in yeast. All

expression plasmids produced in this thesis were generated following the same general procedure. Amplicons were generated from appropriate templates, either plasmid (pM2-GAL4-*Ci*VDR/PXRα, *C. intestinalis* VDR/PXRα) or cDNA (*B. schlosseri* VDR/PXRα) with the following reaction conditions: (i) pGAL4.VP16, pGAL4.*Ci*LBD, pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Ci*LBD.VP16: 1x BioMix (BioLine, London, U.K.); 94°C/2 min., x1 cycle; 94°C/30 sec., 55°C/30 sec., 72°C/90 sec. x35 cycles; 72°C/5 min., x1 cycle; 15°C hold and; (ii) pGAL4.*Bs*LBD and pGAL4.*Bs*LBD.VP16: 1x Sahara Mix (BioLine); 94°C/10 min., x1 cycle; 94°C/30 sec., 45°C/30 sec., ramp 45°C–72°C at 0.2°C/sec., 72°C/2 min., x5 cycles; 94°C/30 sec., 55°C/30 sec., 72°C/2 min., x35 cycles; 72°C/7 min., x1 cycle; 15°C hold. Primers used for each construct are summarised in Appendix 12 and each included the restriction enzyme cleavage sites required for sub-cloning. Because amplicon ends are often refractory to restriction enzyme digestion the amplicons were first separated in agarose gels. Amplicons were gel-extracted (Zymoclean™ Gel DNA Recovery Kit, Zymo Research, Irvine, CA, U.S.A.) and ligated into the T-tailed cloning vector pGemT-easy (Promega, Madison, WI, U.S.A.) before transformation into competent DH5α *E. coli* cells. Plasmids were purified using a commercial kit (High Pure Plasmid Isolation Kit, Roche Diagnostics, Penzberg, Germany) and screened for appropriate inserts by Sanger sequencing (Massey Genome Service, Palmerston North, New Zealand). Subsequently, the plasmid inserts were removed by digestion with an appropriate pair of restriction enzymes (Roche Diagnostics; Appendix 12). The receiving expression plasmid, either pGBKT7 or its derivative of pGAL4.VP16 (Appendix 12), was double digested with an appropriate pair of restriction enzymes and ligated with the digested amplicon using T4 DNA ligase (Roche Diagnostics). Plasmids were transformed into DH5α *E. coli* and transformants selected on luria broth (LB) agar containing kanamycin (25 µg/ml). Plasmids were purified and insert identity and junction sequences confirmed by using sequencing primers flanking the MCS of pGBKT7: 5'–GCCGTCACAGATAGATTGGC–3' (anneals at coordinates 1077–1096 of pGBKT7) and 5'–TTCGCCCGGAATTAGCTTGG–3' (anneals at coordinates 1416–1397 of pGBKT7). For many of the plasmid constructs the base vector used (pGAL4.VP16) was derived from pGBKT7 and encodes 68 residues of a generic activation domain from the herpes simplex virus protein 16

(VP16; Louvion *et al*. 1993; Appendix 12 and 13). The fusion proteins encoded by the various constructs used in this study are shown schematically in Figure 3.2 and more details are given in Appendix 13.

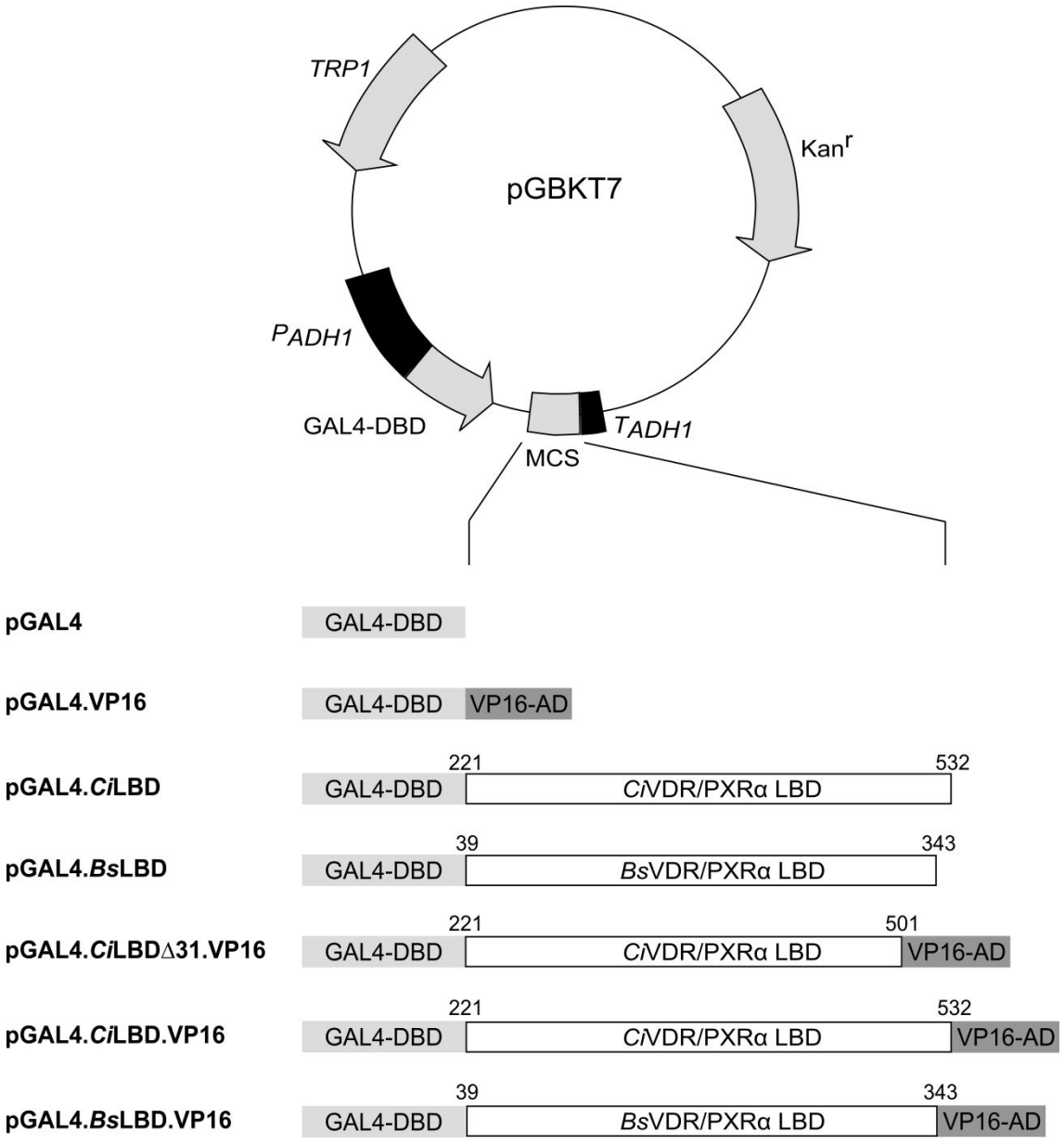

Figure 3.2 Schematic of fusion genes/chimeric proteins generated in this study.

The base vector used for expression was pGBKT7 (Catalogue No. 630443, Clontech). Constitutive transcription of fusion genes is driven by the *alcohol dehydrogenase 1* (*ADH1*) promoter (P_{ADH1}) and terminated by the *ADH1* transcription termination signal (T_{ADH1}). The pGBKT7 vector replicates autonomously in both *Escherichia coli* and *Saccharomyces cerevisiae* and carries a kanamycin resistance (Kan^R) gene for

selection in *E. coli* and the nutritional marker gene *TRP1* for selection in *trp1* yeast strains. The pGBKT7 vector encodes 147 residues of the GAL4 DNA-binding domain (GAL4-DBD) adjacent to a multiple cloning site (MCS). The amino acid residue numbering for the *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα) ligand-binding domains (LBDs) corresponds to that of the predicted proteins of NP_001071847 and AHB39790, respectively. Some of the plasmid constructs encode 68 residues of a generic activation domain (AD) from the herpes simplex virus protein 16 (VP16; Louvion *et al.* 1993). Further details of the cloning procedure used and the corresponding chimeric protein sequences are given in Appendix 12 and 13.

3.2.4 Yeast transformation

The *S. cerevisiae* host strain PJ69-4A (*MATa*, *trp1-901*, *leu2-3 112*, *ura3-52*, *his3-200*, *gal4*Δ, *gal80*Δ, *GAL2-ADE2*, *LYS2::GAL1-HIS3*, *met2::GAL7-lacZ*; James *et al*. 1996) was transformed using the lithium acetate method (Gietz & Schiestl 2007) with tryptophan prototrophic transformants selected on solid selection media lacking tryptophan (2% (w/v) agar, 0.67% (w/v) Difco yeast nitrogen base without amino acids, 2% (w/v) acid-hydrolysed casamino acids, 2% (w/v) D(+) glucose, 120 µg/ml adenine, 20 µg/ml uracil, 20 µg/ml L-methionine and 20 µg/ml L-histidine). Single colony transformants were streaked out twice on selective media agar plates to ensure that the transformed strains used were clonal. The resulting strains were subsequently propagated on GM agar plates. The seven strains used to prepare starter stocks corresponded to each of the seven plasmids generated in this work (Figure 3.2, Appendix 12 and 13).

3.2.5 Preparation of yeast starter stocks

Yeast strains were grown in 50 ml of liquid GM in 300 ml conical flasks (30°C, 230 rpm) for 24–48 h until reaching an OD_{620} of approximately 1 (Nano Photometer, Implen, Munich, Germany). Culture aliquots of 0.5 ml were mixed

with 0.5 ml of 30% (v/v) sterile glycerol (final glycerol concentration 15% (v/v)) and frozen on dry ice before storage at -70°C.

3.2.6 Yeast bioassay procedure

Freshly prepared GM (7 ml in 50 ml centrifuge tubes, CentriStar™, Corning Inc., NY, U.S.A.) was inoculated with 1 ml of 'yeast starter stock' (Section 3.2.5, final glycerol concentration 2.1% (v/v)) and incubated (30°C, 230 rpm) for 24–48 h until the OD_{620} was approximately 1 (Nano Photometer, Implen). The cultures were then diluted in GM to OD_{620} of approximately 0.1 and this mixture was termed the seeded assay medium (SAM). A twelve step dilution series of chemicals to be tested was prepared in sterile 1.7 ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) using analytical grade ethanol as the solvent (Section 3.2.1). Aliquots (2 µl) of each dilution were pipetted onto the floor of the wells of a 96-well microtitre plate (sterile, flat bottom polystyrene plates with lid, Corning Inc.). To facilitate inter-plate comparisons, each plate contained two columns of control wells (column one, rows A–H: wells containing SAM only; column two, rows A–H: containing 2 µl ethanol solvent plus SAM; Figure 3.3).

Based on published mean effective concentrations (EC_{50} values) and *in vivo* toxicity data, a wide concentration range (0–1 mM) of test compounds was selected for the initial bioassays. Each compound was tested at least twice in separate assays. These data were then used to identify a concentration range that allowed detection of induction of β-galactosidase enzymatic activity in the bioassay while being non-toxic to the yeast cells themselves. Using the identified concentration range, all yeast bioassays were performed in pseudo-triplicates (triplicate measurements within the same assay).

Aliquots of SAM (150 µl) were added to all wells and the plates shaken (600 rpm, 2 min., MSI Minishaker, IKA, Hamburg, Germany) before incubation (30°C) for 24 h to allow ligand-dependent activation of *lacZ* gene expression (De Boever *et al.* 2001). After 24 h incubation, chlorophenol red-β-D-

galactopyranoside (CPRG, Roche Diagnostics) was dissolved in GM and added to each well to a final concentration of 0.1 mg/ml. Plates were shaken (600 rpm, 2 min.) before further incubation (30°C). After 24 h and 48 h the plates were sealed with a sterile plate seal (Classic SealPlate® Sealing Film, Excel Scientific, Victorville, CA, U.S.A.), shaken (600 rpm, 2 min.), and briefly centrifuged (300 rpm, 10 sec., 5810R centrifuge, Eppendorf AG) before OD_{540} and OD_{620} measurements (2300 EnSpire™ Multilabel Reader, Perkin Elmer, Turku, Finland) were taken.

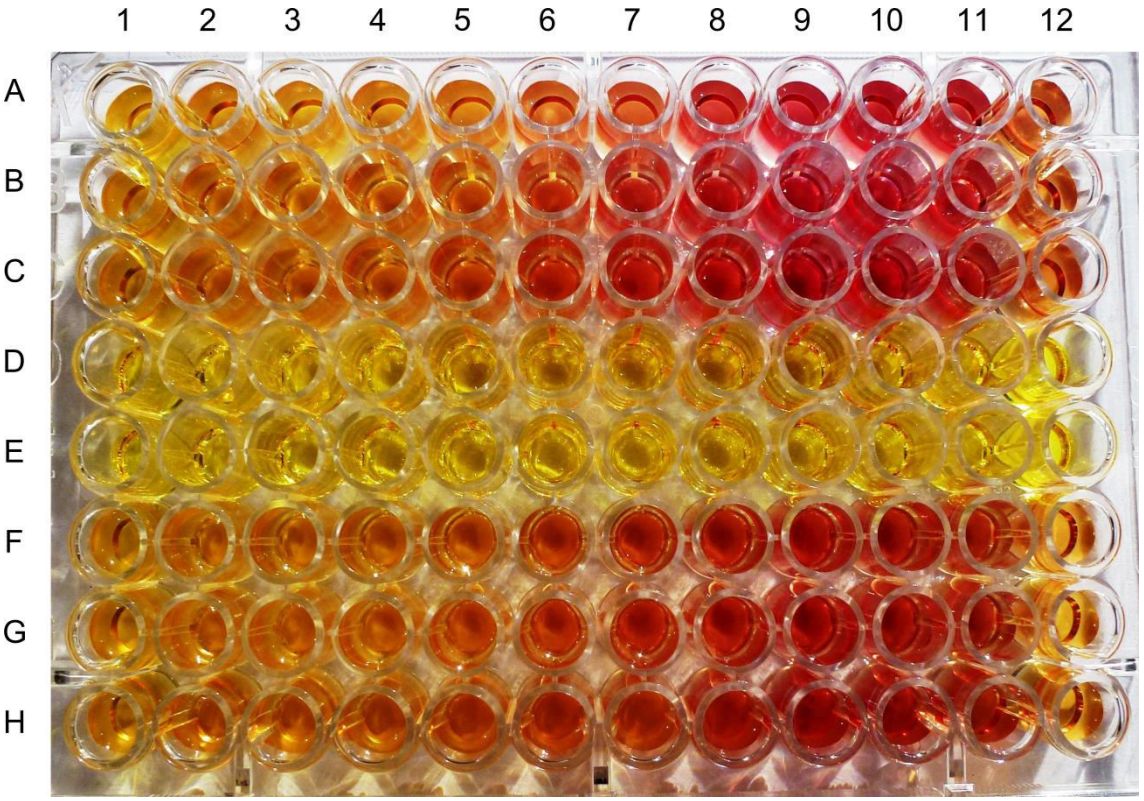

Figure 3.3 Example of a 96-well plate format used in the tunicate yeast bioassays.

Plate showing induction of β-galactosidase activity in yeast strains exposed to *n*-butyl-*p*-aminobenzoate. Yeast strains expressing plasmids pGAL4.*Bs*LBD.VP16 (rows A–C), pGAL4 (rows D–E) and pGAL4.*Ci*LBD.VP16 (rows F–H) were exposed to various concentrations (1–400 µM) of *n*-butyl-*p*-aminobenzoate (columns 3–12). Column 1 contains seeded assay medium (SAM) only and column 2 contains 1% ethanol solvent plus SAM.

3.2.7 Statistics

To correct for variations in cell densities, the raw OD_{540} values were normalised using the equation of Routledge and Sumpter (1996):

$$\mathrm{OD}_{540}\ (\mathrm{corrected}) = \mathrm{Sample\ OD}_{540} - \left(\mathrm{Sample\ OD}_{620} - \mathrm{Blank\ OD}_{620}\right). \quad (1)$$

Blank OD_{620} values correspond to wells containing ethanol solvent and SAM, while sample OD_{540} and sample OD_{620} values correspond to wells containing the test chemicals (Section 3.2.6). Sample OD_{620} values were used to assess general toxicity of test compounds towards yeast cells. The median effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) were determined from four-parameter sigmoidal concentration-response curves using GraphPad Prism Version 6.03 (GraphPad Software, La Jolla, CA, U.S.A., http://graphpad.com) following Routledge & Sumpter (1996) and Karimullina *et al*. (2012).

Intra-plate variability was quantified by calculating the coefficient of variance (CV, %). For each compound concentration the mean ($\bar{x}$) and the standard deviation (SD) for the three replicate measurements were determined. The CV for each compound concentration was calculated as follows:

$$\mathrm{CV} = \frac{\mathrm{SD}}{\bar{x}} \times 100\ \%. \quad (2)$$

The average of the individual CVs is reported as the intra-assay CV for each yeast strain (Yang *et al.* 2014).

To assess plate-to-plate consistency, inter-plate CVs were calculated using a known *C. intestinalis* VDR/PXRα LBD agonist, *n*-butyl-*p*-aminobenzoate (Ekins *et al.* 2008). Assays were run in triplicates on three different plates. The plate means for each *n*-butyl-*p*-aminobenzoate concentration were calculated and then used to calculate the overall mean, SD and CV. The overall CV for each concentration was calculated as follows:

$$\mathrm{CV} = \frac{\text{SD of plate } \bar{x}}{\bar{x} \text{ of plate } \bar{x}} \times 100\ \% \quad (3)$$

The average of the overall CVs for each compound concentration is reported as the inter-assay CV (Yang *et al.* 2014).

3.3 Results and discussion

3.3.1 Development of the bioassay yeast strains

The objective of this chapter was to develop recombinant yeast bioassays in which β-galactosidase enzymatic activity (encoded by *lacZ*) was induced by ligands that activate tunicate xenobiotic receptors. There are two main components to the yeast bioassays: the yeast host strain and the plasmid constructs transformed into the yeast.

The yeast (*S. cerevisiae*) host strain selected for this work was PJ69-4A (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*; James *et al.* 1996). This strain is characterised by multiple genotypic features making it a suitable host strain: (i) *trp1-901*: complementation of *trp1-901* by the *TRP1* gene encoded by pGBKT7-derived plasmids allows selection for tryptophan prototrophic transformants on media lacking tryptophan; (ii) *gal4Δ, gal80Δ*: deletion mutations in the *GAL4* and *GAL80* nuclear genes render the endogenous GAL4/GAL80 galactose-sensitive induction pathway non-functional (Traven *et al.* 2006) and; (iii) *met2::GAL7-lacZ*: this modification places a *lacZ* gene under the control of a GAL4-regulated promoter. The *lacZ* reporter gene product, *E. coli* β-galactosidase enzyme, catalyses hydrolysis of the colorimetric substrate CPRG without the need for cell lysis, thus allowing repeated, non-lethal measurements of the yeast bioassay's output.

All seven plasmids generated in this study encode chimeric proteins combining one or more of three basic elements: (i) a GAL4-DBD; (ii) a tunicate VDR/PXRα

orthologue LBD and; (iii) a VP16-AD, derived from herpes simplex virus protein 16 (Louvion *et al.* 1993). While the sequences encoding the GAL4-DBD and VP16-AD are well established from previous studies (Louvion *et al.* 1993), determination of the appropriate tunicate VDR/PXRα orthologue LBD sequences, to be expressed in this study, required careful consideration. Computer prediction programmes (e.g. Simple Modular Architecture Research Tool (SMART); Schultz *et al*. 1998; Lubec *et al*. 2005; Letunic *et al*. 2012) appeared to underestimate the size of xenobiotic receptor LBDs (Chapter Two, Section 2.4.1). To address this problem, the tunicate VDR/PXRα LBD domain was defined as comprising residues just C-terminal of the predicted DBD through to the C-terminal end of the predicted open reading frame. This is the same region used previously for expression of the *C. intestinalis* VDR/PXRα LBD domain in mammalian cell lines (Ekins *et al.* 2008; Fidler *et al.* 2012).

The seven plasmids used in this study were as follows. Plasmid pGAL4 is simply the base vector pGBKT7 encoding the GAL4-DBD domain and a short, non-functional C-terminal sequence (Figure 3.2, Appendix 13). Plasmid pGAL4.VP16 encodes the GAL4-DBD fused to the VP16-AD which has been found useful for coupling ligand-activated NRs in yeast to activation of reporter genes (Louvion *et al*. 1993; Figure 3.2, Appendix 12 and 13). Together, pGAL4 and pGAL4.VP16 are the negative control plasmids that control for reporter gene expression changes in the absence of any tunicate VDR/PXRα LBD domain sequences. Plasmid pGAL4.*Ci*LBD expresses a fusion protein consisting of the GAL4-DBD fused to the *C. intestinalis* VDR/PXRα LBD (residues 221–532 of NP_001071847; Figure 3.2, Appendix 12 and 13). Plasmid pGAL4.*Ci*LBD.VP16 encodes the same region of the *C. intestinalis* VDR/PXRα LBD as pGAL4.*Ci*LBD but, in addition, encodes the VP16-AD on the C-terminus of the expressed protein (Figure 3.2, Appendix 12 and 13). Plasmid pGAL4.*Ci*LBDΔ31.VP16 is similar to pGAL4.*Ci*LBD.VP16 but the LBD is 31 residues shorter with the C-terminal flank of the LBD being that predicted by SMART (i.e. residues 221–501 of NP_001071847; Letunic *et al*. 2012; Figure 3.2, Appendix 12 and 13). Plasmid pGAL4.*Bs*LBD expresses the *B. schlosseri* VDR/PXRα LBD residues 39–343 of AHB39790 (Figure 3.2, Appendix 12 and 13). Plasmid pGAL4.*Bs*LBD.VP16 encodes the same region of the *B. schlosseri*

VDR/PXRα LBD as pGAL4.*Bs*LBD but, in addition, has the VP16-AD on the C-terminus of the expressed fusion protein (Figure 3.2, Appendix 12 and 13).

In total, five plasmids were generated that encoded chimeric proteins containing both the GAL4-DBD and a tunicate VDR/PXRα LBD (Figure 3.2, Appendix 13). Two of these plasmids, pGAL4.*Ci*LBD and pGAL4.*Bs*LBD, expressed fusion proteins lacking the VP16-AD (Figure 3.2, Appendix 13). For these chimeric proteins to function, their native ligand-dependent activation domain (AF-2) needs to function within the nuclear milieu of yeast cells. As previous studies have shown that the AF-2 domain of at least some vertebrate NRs functions in yeast (Pham *et al.* 1992; vom Baur *et al.* 1998; Gaudon *et al.* 1999), the two constructs were generated to test if this was also observed in the tunicate VDR/PXRα receptors.

3.3.2 Yeast bioassay media optimisation

The permissiveness of vertebrate PXR LBDs with respect to cognate ligand structures (Ekins *et al.* 2008; Fidler *et al.* 2012) raised concerns that some yeast growth media components might act as tunicate VDR/PXRα LBD receptor ligands, thereby compromising the yeast bioassay. More specifically, two amino acids (L-tyrosine and L-phenylalanine) present in the original yeast media of Routledge and Sumpter (1996) and Beresford *et al*. (2000) had aromatic structures suggesting that they might be potential tunicate VDR/PXRα LBD ligands. While preliminary experiments established that neither of these amino acids induced β-galactosidase enzymatic activity from any of the seven strains tested (data not shown), both amino acids were omitted from the yeast growth media as a precaution.

In the course of preliminary experiments on media composition, it was noted that strains carrying three plasmids (pGAL4.*Ci*LBD.VP16, pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Bs*LBD.VP16) grew faster in the absence of L-histidine than strains carrying any of the other four plasmids. To investigate this observation, all seven yeast strains were grown in media lacking L-histidine or supplemented with L-histidine at one of three concentrations, and cell density

(OD_{620}) was measured over time (0–63 h; Figure 3.4). In the absence of L-histidine in the media, the three strains carrying plasmids that encode a tunicate VDR/PXRα LBD combined with the VP16-AD grew much faster than the other four strains (Figure 3.4A). With increasing concentrations of exogenously supplied L-histidine, the degree of differential growth between the seven strains diminished (Figure 3.4B–D). In media containing both 200 and 322 µM L-histidine there was no observed difference in OD_{620} values between any of the seven strains at 63 h of incubation (Figure 3.4C–D). The observed histidine-dependent differential growth of the yeast strains can be explained by noting that the yeast host strain genotype (*MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*) contains both the *his3-200* mutant allele and a functional *HIS3* gene under the control of the GAL4-regulated *GAL1* promoter. The three chimeric proteins encoded by the plasmids pGAL4.*Ci*LBD.VP16, pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Bs*LBD.VP16 appeared to enhance transcription of the *HIS3* gene from its GAL4-activated *GAL1* promoter, leading to L-histidine biosynthesis and enhanced strain growth in L-histidine-limited media (Figure 3.4A, B). This induction of the GAL4-activated *GAL1* promoter, even in the absence of any experimentally added inducing compound(s), suggests low background levels of constitutive activity by the three chimeric proteins. Alternatively, the three chimeric proteins may be activated by either endogenously synthesised yeast compounds or by yeast growth media components. In contrast, the GAL4-regulated *GAL1* promoter of *HIS3* was inactive in the four yeast strains carrying plasmids pGAL4, pGAL4.VP16, pGAL4.*Ci*LBD and pGAL4.*Bs*LBD leading to little or no growth in L-histidine-limited media (Figure 3.4A).

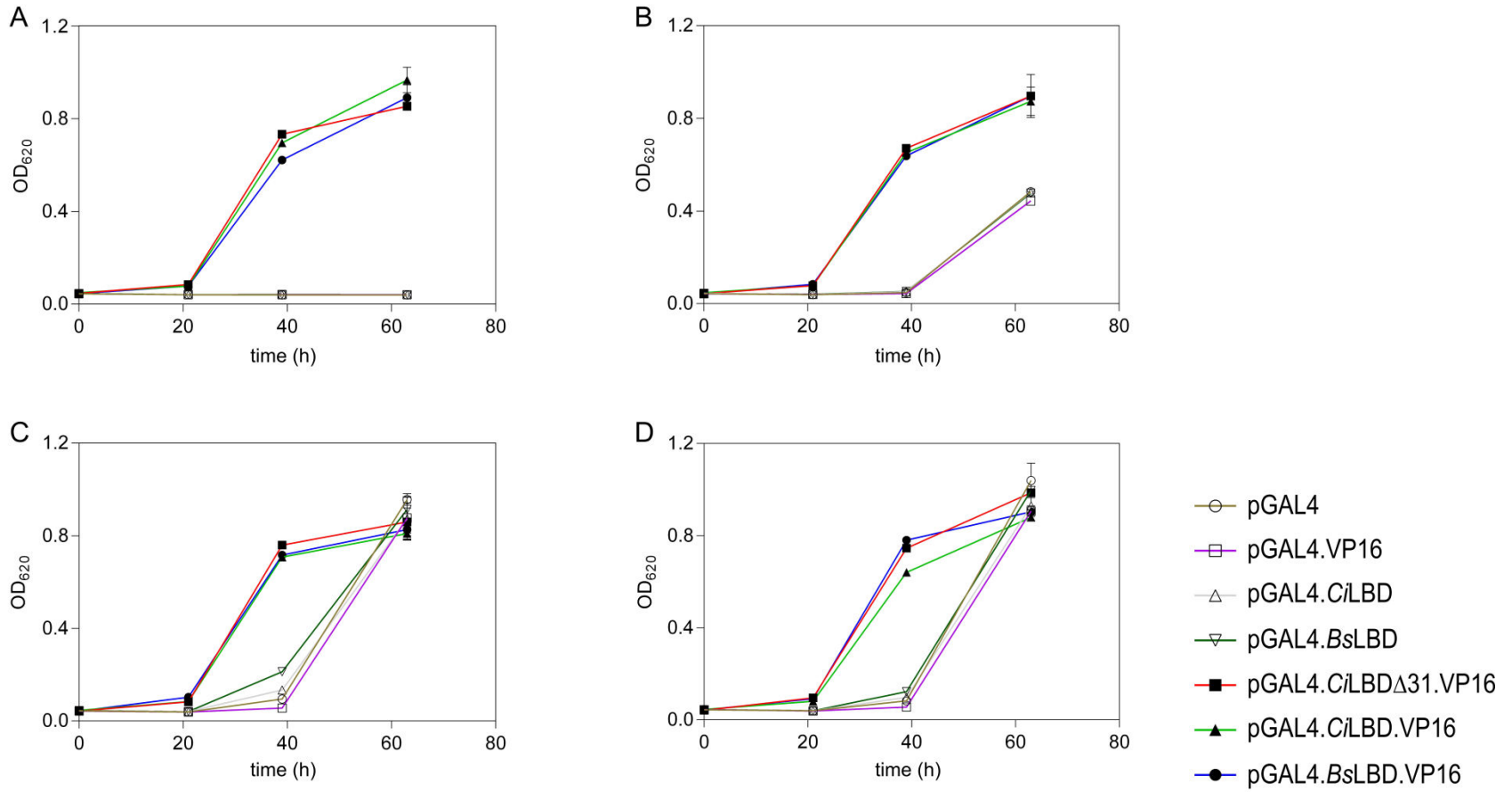

Figure 3.4 Media concentrations of L-histidine influence the growth rate of three recombinant yeast strains.

Yeast strains carrying the pGBKT7-based expression plasmids described in Section 3.3.1 were grown in liquid growth media with differing concentrations of L-histidine: (**A**) 0 µM, (**B**) 10 µM, (**C**) 200 µM and (**D**) 322 µM. Cell density was quantified by OD_{620} values and measured at four time points (0, 21, 39 and 63 h) following culture establishment. Data points represent means of n = 3 replicates. Error bars show ± one standard deviation.

To explore this interpretation further, β-galactosidase induction by an established *C. intestinalis* VDR/PXRα agonist, *n*-butyl-*p*-aminobenzoate (Ekins *et al.* 2008), was measured in media containing four concentrations of L-histidine (Figure 3.5). The response curves of the three strains showing *n*-butyl-*p*-aminobenzoate-dependent β-galactosidase induction (i.e. carrying plasmids pGAL4.*Ci*LBD.VP16, pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Bs*LBD.VP16) became progressively less sigmoidal with increasing concentrations of media-supplied exogenous L-histidine (Figure 3.5A–D). In addition, the four strains that showed no ligand-dependent induction of β-galactosidase activity exhibited elevated background levels of β-galactosidase enzymatic activity at higher L-histidine concentrations (Figure 3.5A–D). As the *E.coli* β-galactosidase protein (http://uniprot.org/uniprot/P00722), encoded by the *lacZ* reporter gene, contains multiple L-histidine residues it appeared that the β-galactosidase enzymatic activity induction curves generated in this study are a summation of the ligand-dependent activation of two GAL4-regulated promoters/genes in the host strain genome, specifically *GAL7-lacZ* and *GAL1-HIS3* (Traven *et al.* 2006). This suggests that the yeast bioassay developed measures the 'output' of β-galactosidase enzymatic activity of a single ligand-activated chimeric protein controlling two promoters, both reducing background signal and creating a more sigmoidal induction response (Figure 3.5A–D).

To provide a compromise between the need for exogenous L-histidine, essential for growth of some yeast strains (pGAL4, pGAL4.VP16, pGAL4.*Ci*LBD and pGAL4.*Bs*LBD) and the requirement to minimise the background (i.e. not ligand-induced β-galactosidase enzymatic activity), the seven yeast strains were first grown in the presence of L-histidine to generate the yeast starter stock (Section 3.2.5). The strains carrying plasmids pGAL4, pGAL4.VP16, pGAL4.*Ci*LBD and pGAL4.*Bs*LBD were supplied with 322 µM L-histidine and strains carrying plasmids pGAL4.*Ci*LBD.VP16, pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Bs*LBD.VP16 were supplied with 10 µM L-histidine. No exogenous L-histidine was provided in the SAM (Section 3.2.6) which was used for the yeast bioassay itself.

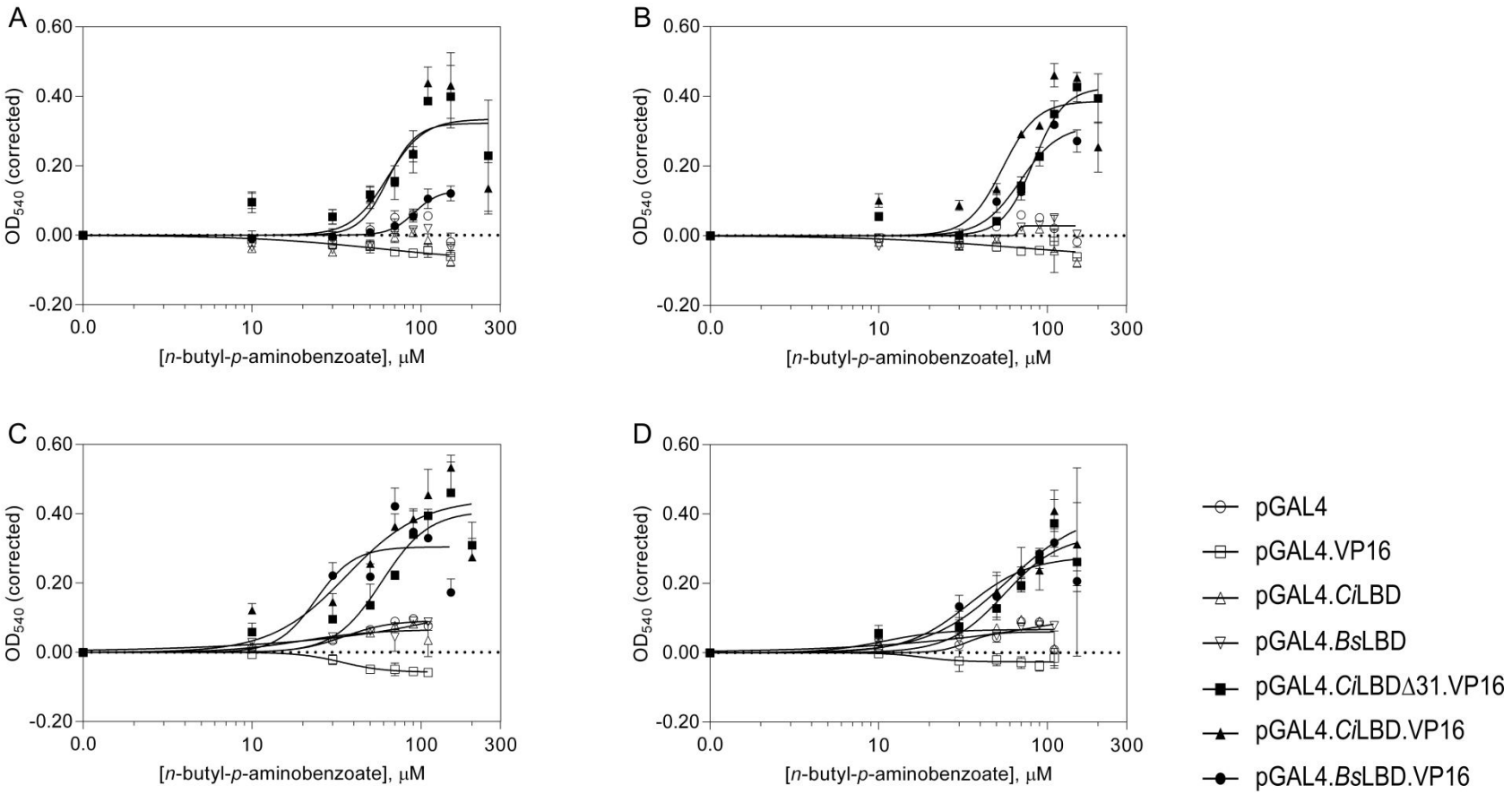

Figure 3.5 Influence of L-histidine concentrations on background activity of β-galactosidase.

Yeast strains were exposed to *n*-butyl-*p*-aminobenzoate over a range of concentrations (0–300 µM) in growth media containing four concentrations of L-histidine: (**A**) 0 µM, (**B**) 10 µM, (**C**) 200 µM and (**D**) 322 µM. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined as described in Section 3.2.6. Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

3.3.3 Activity of synthetic toxicants in the yeast bioassays

Having optimised the media conditions for the recombinant yeast strains used in the bioassays, the ability of *n*-butyl-*p*-aminobenzoate and three additional synthetic chemicals (carbamazepine, bisphenol-A (BPA) and *p*-aminobenzoic acid) to induce β-galactosidase enzymatic activity were investigated (Table 3.1, Figure 3.6).

Two of the synthetic chemicals tested (*n*-butyl-*p*-aminobenzoate and carbamazepine) were positive controls for the yeast bioassays because they had previously been shown to activate the *C. intestinalis* VDR/PXRα LBD when expressed as a fusion gene in mammalian cell lines (Ekins *et al.* 2008). *N*-butyl-*p*-aminobenzoate and carbamazepine both induced β-galactosidase enzymatic activity from the three yeast strains carrying plasmids encoding a tunicate VDR/PXRα LBD fused to the VP16-AD (pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16; Figure 3.6A–B, Table 3.1). Strains carrying the two negative control plasmids, pGAL4 and pGAL4.VP16, showed no evidence of induced β-galactosidase enzymatic activity, nor did the two strains encoding GAL4.VDR/PXRα LBD fusion proteins lacking the VP16-AD (pGAL4.*Ci*LBD, pGAL4.*Bs*LBD; Figure 3.6A–B). For some strains there was evidence of reduced β-galactosidase enzymatic activity at higher concentrations of both *n*-butyl-*p*-aminobenzoate and carbamazepine (Figure 3.6A, B). This suppression effect does not appear to be an antagonistic effect mediated through the tunicate VDR/PXRα LBD as it is apparent in the negative control strains, being most prominent with plasmid pGAL4.VP16 at high concentrations of carbamazepine (Figure 3.6B). Examination of the OD_{620} values from the yeast bioassays shows a general toxic effect of *n*-butyl-*p*-aminobenzoate and carbamazepine towards yeast cells, particularly at high concentrations (Appendix 14). The sensitivity of the pGAL4.*Ci*LBD.VP16 yeast bioassay (*n*-butyl-*p*-aminobenzoate: EC_{50} = 55 µM; carbamazepine: EC_{50} = 6.2 µM; Table 3.1) was comparable to that of the mammalian cell line bioassay expressing *C. intestinalis* VDR/PXRα (*n*-butyl-*p*-aminobenzoate: EC_{50} = 16.5 µM; carbamazepine: EC_{50} >10 µM; Ekins *et al.* 2008). The yeast strain expressing the shorter version of the *Ci*VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16) was

slightly less sensitive to both *n*-butyl-*p*-aminobenzoate and carbamazepine than strains expressing the longer version (pGAL4.*Ci*LBD.VP16) as indicated by the shape of the inductive response and EC_{50} values (Figure 3.6A–B, Table 3.1).

Two additional synthetic chemicals (*p*-aminobenzoic acid and BPA), with planar structures and at least one hydrogen bond acceptor (Figure 3.1), were tested in the yeast bioassays (Table 3.1, Figure 3.6C–D). Both induced positive responses although these were non-sigmoidal. The *B. schlosseri* VDR/PXRα LBD-based yeast bioassay (pGAL4.*Bs*LBD.VP16) was less sensitive than the *C. intestinalis* VDR/PXRα equivalent (Figure 3.6C, D, Table 3.1). Activity of *p*-aminobenzoic acid in the mammalian cell line bioassay expressing *Ci*VDR/PXRα had been noted previously although the yeast bioassay appears to be more sensitive (*p*-aminobenzoic acid: yeast EC_{50} = 3 µM; mammalian EC_{50} = 10–30 µM; M. Krasowski, *personal communication*). Bisphenol-A, an estrogenic endocrine disruptor (Olea *et al.* 1996; Welshons *et al.* 2003), has been identified as a potent toxicant when tested on tunicate larvae (Mansueto *et al.* 2011; Cangialosi *et al.* 2013) with low concentrations (3–10 µM) of BPA affecting swimming, hatching and normal development of *C. intestinalis* larvae (Matsushima *et al.* 2013). Given the comparable sensitivities of the pGAL4.*Ci*LBD.VP16-based yeast bioassays (EC_{50} = 9.2 µM; Table 3.1) these yeast bioassays may, at least in some contexts, provide cheap and consistent proxies for assessing the effects of environmental contaminants on invertebrate members of coastal ecosystems (Stewart *et al.* 2014). To further establish the validity of this idea, it would be useful to systematically test synthetic chemicals known to be toxic to *C. intestinalis* and *B. schlosseri* in the yeast bioassays (Bellas *et al.* 2003).

The failure of yeast strains carrying plasmids pGAL4.*Ci*LBD and pGAL4.*Bs*LBD to display ligand-dependent induction of β-galactosidase enzymatic activity indicates that any transcription AF-2 domain within a tunicate VDR/PXRα LBD is non-functional in yeast cells (Berry *et al.* 1990; Louvion *et al.* 1993). Future yeast bioassays based around the template described in this thesis should incorporate a C-terminal VP16-AD domain onto any fusion proteins generated.

Table 3.1 Activation of the tunicate yeast bioassays by four synthetic chemicals.

Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in µM. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in ethanol.

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
N-butyl-*p*-amino-benzoate [a]	Local anaesthetic	Sigma-Aldrich (B7753)	68 (64–74)	16	55 (48–63)	11	57 (47–69)	14
Carbamazepine [b]	Anticonvulsant	Sigma-Aldrich (C4024)	7.1 (4.9–9.9)	18	6.2 (5.3–7.3)	7	7.0 (4.9–9.8)	21
P-aminobenzoic acid [b]	Supplement	Sigma-Aldrich (A9878)	1.8 (1.2–2.9)	14	3.0 (1.1–8.8)	25	~191 (DNC)	89
Bisphenol-A [b]	EDC	Sigma-Aldrich (B1760)	5.3 (1.8–15.6)	18	9.2 (4.9–17.5)	14	10 (0–4108)	85

Abbreviations: EDC, endocrine disrupting chemical.

[a] Strains were incubated for 24 hours.

[b] Strains were incubated for 48 hours.

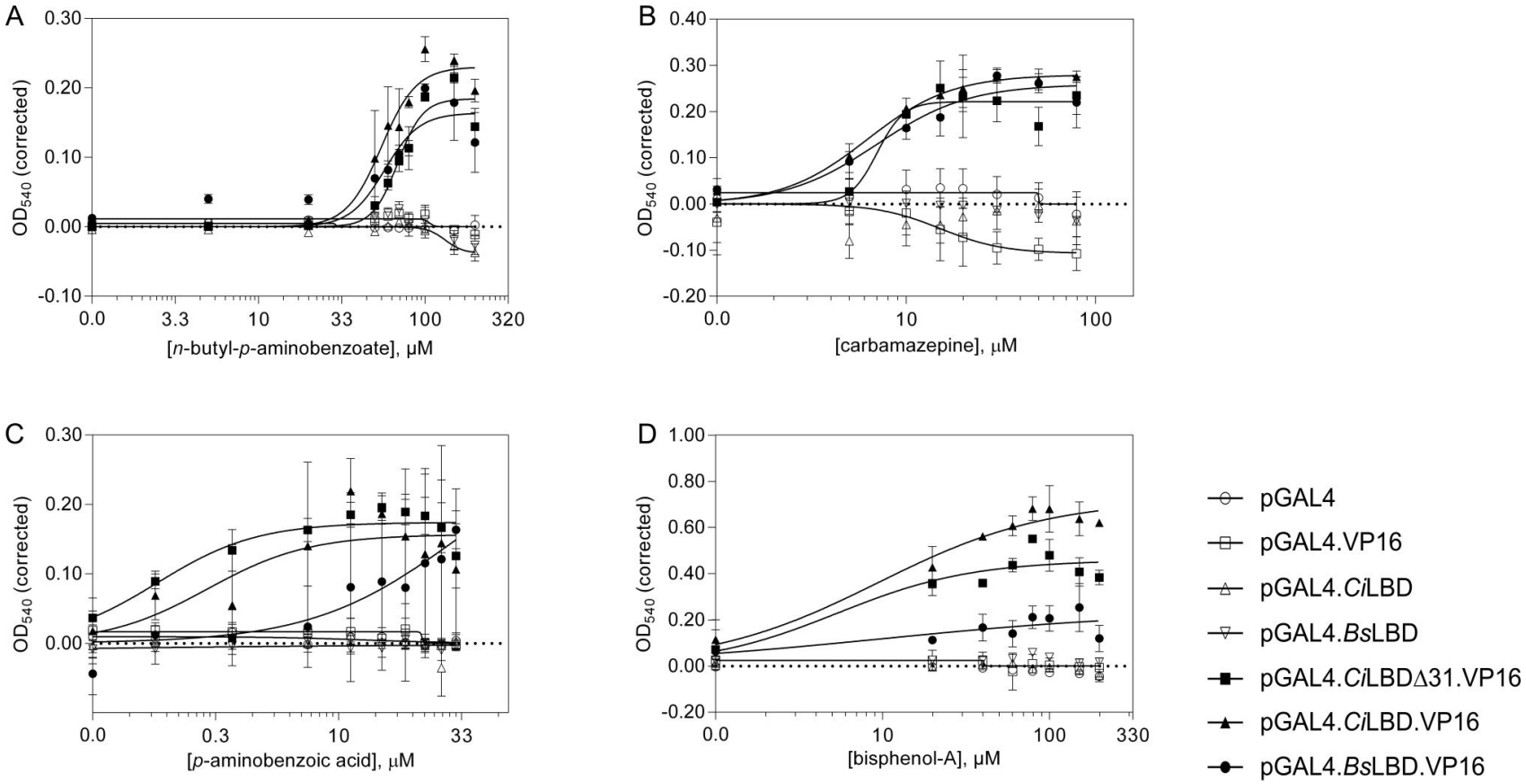

Figure 3.6 Activities of four synthetic chemicals tested in the yeast bioassays.

Yeast strains carrying seven pGBKT7-based expression vectors were exposed to a range of concentrations of: (**A**) *n*-butyl-*p*-aminobenzoate, (**B**) carbamazepine, (**C**) *p*-aminobenzoic acid and (**D**) bisphenol-A. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 24 h or 48 h (Table 3.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

3.3.4 Activity of natural microalgal biotoxins in the yeast bioassays

Pursuing the hypothesis that the ecologically relevant ligands of tunicate VDR/PXRα receptors include those toxins naturally present in a marine filter-feeder's diet, three established microalgal biotoxins (okadaic acid, PTX-11 and portimine; Figure 3.1) were tested in the yeast bioassays. All three microalgal biotoxins induced β-galactosidase enzymatic activity from those strains carrying plasmids encoding a tunicate VDR/PXRα LBD fused to the VP16-AD: pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 (Table 3.2, Figure 3.7A–C). In contrast, the yeast strains carrying the two negative control plasmids, pGAL4 and pGAL4.VP16, showed no evidence of induced β-galactosidase enzymatic activity nor did the two strains encoding GAL4.VDR/PXRα LBD fusion proteins lacking a C-terminal VP16-AD domain (Figure 3.7A–C). There was no indication of toxicity of microalgal biotoxins towards the yeast cells within the concentration ranges tested (Figure 3.7A–C).

Two freshwater cyanobacterial toxins (microcystin-RR and anatoxin-A) were also tested in the yeast bioassays. They were considered to be 'negative controls' as being of freshwater origin, they would not have been encountered by marine tunicates over evolutionary time. As expected both were inactive in the yeast bioassays (Table 3.2).

Identification of portimine as an activator of both tunicate VDR/PXRα LBDs tested (Table 3.2) is consistent with its reported toxicity when tested using mammalian cells *in vitro* (LC_{50} = 2.7 nM) suggesting that portimine may be toxic towards a wide range of chordates— including tunicates (Selwood *et al.* 2013). The yeast strain expressing the shorter version of the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16) was more strongly activated by portimine than the strain expressing the longer version (pGAL4.*Ci*LBD.VP16) as indicated by the plateau levels of β-galactosidase production (Figure 3.7C). This difference was not reflected in EC_{50} values (Table 3.2). In contrast, the yeast strain expressing the shorter version of the *C. intestinalis* VDR/PXRα LBD was less strongly activated by okadaic acid and PTX-11 as reflected in plateau levels of β-galactosidase activity (Figure 3.7A–B). The EC_{50} values for the pGAL4.*Ci*LBDΔ31.VP16 strain were lower than for the pGAL4.*Ci*LBD.VP16

strain for both okadaic acid and PTX-11 (Table 3.2). The EC_{50} values for activation of the full-length *C. intestinalis* VDR/PXRα LBD by okadaic acid (pGAL4.*Ci*LBD.VP16 EC_{50} = 27 nM; Table 3.2) is comparable with the value reported using mammalian cell line *Ci*VDR/PXRα-based bioassays (EC_{50} = 18.2 nM) supporting the validity of the yeast bioassays (Fidler *et al.* 2012).

Two of the microalgal biotoxins that activated the tunicate VDR/PXRα LBDs in this study (okadaic acid and PTX-11) are produced by dinoflagellate species within the cosmopolitan genus *Dinophysis*, which can reach cell densities of 10^2–10^5 cells/L in coastal marine waters (Reguera *et al.* 2012). Thus, it is to be expected that filter-feeding marine invertebrates will encounter and accumulate such biotoxins through their diet (Sekiguchi *et al.* 2001; Echevarria *et al.* 2012; Roje-Busatto & Ujević 2014). Although the toxicity of microalgal biotoxins towards tunicates, both adult and tadpole, requires more investigation, the yeast bioassay data presented in this chapter is consistent with the speculation that tunicate VDR/PXRα LBDs have adaptively evolved to bind commonly encountered marine biotoxins (Fidler *et al.* 2012). Further investigations are required to assess the toxicity of structurally diverse microalgal biotoxins towards tunicates. These studies would be complemented by determination of microalgal biotoxin EC_{50} values using the yeast bioassays described here.

Table 3.2 Activation of the tunicate yeast bioassays by five algal toxins.

Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in nM. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in ethanol.

Chemical name	**Toxin type**	**Supplier (Catalogue No.)**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
			EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Okadaic acid [a]	Microalgal biotoxin	Sapphire Bioscience (AB120375)	19 (1.1–327)	46	27 (15.4–46.4)	8	16 (8.3–30.4)	8
Pectenotoxin-11 [a]	Microalgal biotoxin	MacKenzie *et al.* 2013	633 (117–3437)	66	883 (134–5834)	35	553 (3.4–90,840)	46
Portimine [a]	Microalgal biotoxin	Selwood *et al*. 2013	143 (114–180)	10	130 (105–162)	12	124 (77–202)	7
Microcystin-RR [a]	Cyanobacterial toxin	DHI Lab Products (PPS-MCRR)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Anatoxin-A [a]	Cyanobacterial toxin	NRC Canada (IMB-CRM-ATX)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC

Abbreviations: DNC, did not compute; NI, no induction of β-galactosidase enzymatic activity.

[a] Strains were incubated for 48 hours.

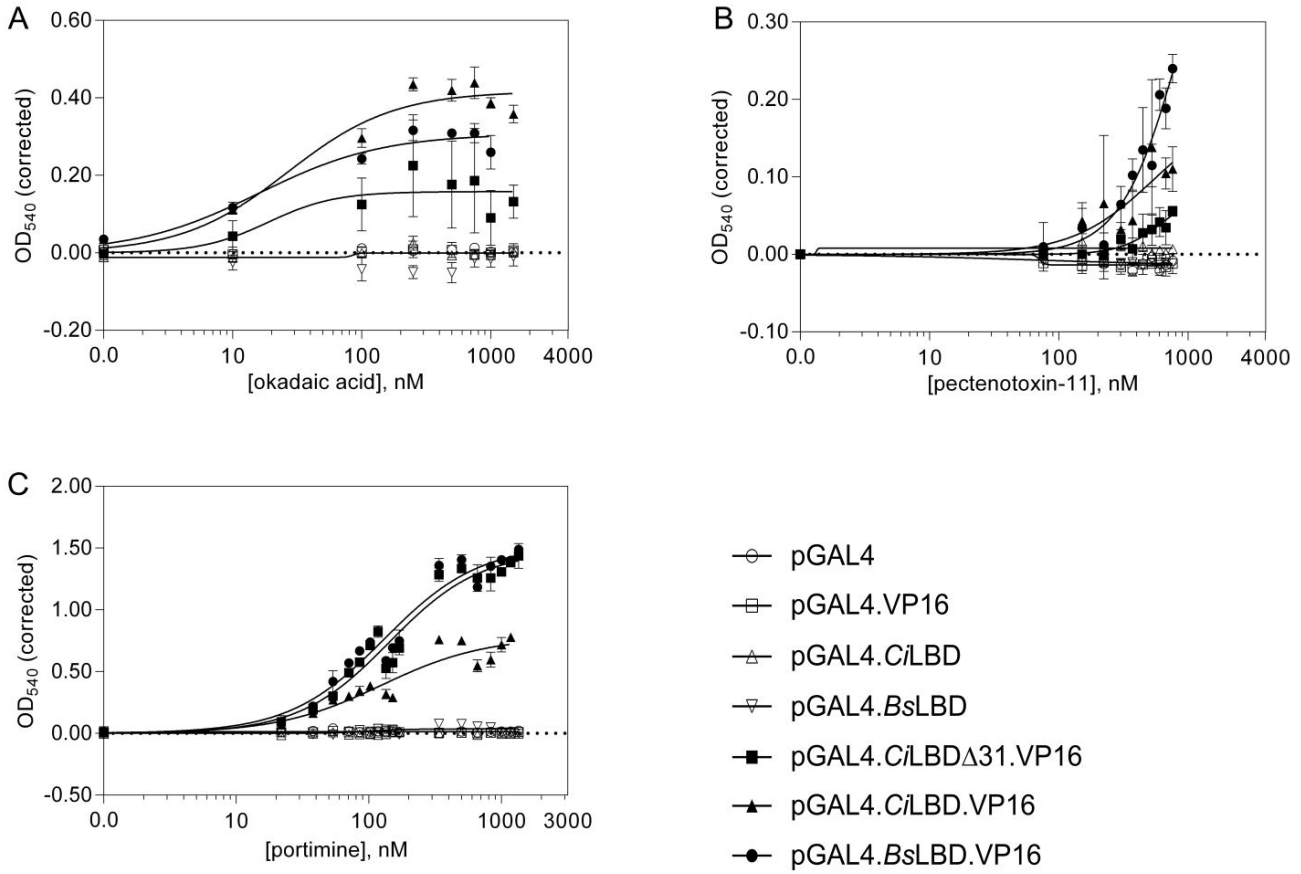

Figure 3.7 Activities of three microalgal biotoxins tested in the yeast bioassays.

Yeast strains carrying seven pGBKT7-based expression vectors were exposed to a range of concentrations of (**A**) okadaic acid, (**B**) pectenotoxin-11 and (**C**) portimine. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h. Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

3.3.5 Characteristics of the yeast bioassays

Precision of the yeast bioassay was determined using the repeatability between three replicate assays of *n*-butyl-*p*-aminobenzoate reported as inter-plate CVs (Section 3.2.7). Coefficients of variance ranged from 4% in positive yeast strains to 30% in negative strains (Table 3.3). The low CV reported for three yeast strains (pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16; CV <5%) indicates that the yeast bioassay test results are consistent and repeatable. The elevated CV values (11–30%) observed in the four negative yeast strains (pGAL4, pGAL4.VP16, pGAL4.*Ci*LBD and pGAL4.*Bs*LBD) can be explained by the lack of induction of β-galactosidase activity in these strains. This causes negative values for some ligand concentrations making the CV calculation difficult, since CVs can only be computed from non-negative values.

Table 3.3 Inter-plate variability of the tunicate yeast bioassays.
Yeast strains carrying the seven pGBKT7-based expression plasmids were exposed to varying concentrations of *n*-butyl-*p*-aminobenzoate (0–200 µM) in three separate bioassays. Triplicate measurements were taken within each bioassay as described in Section 3.2.7. Results are presented as grand mean, standard deviation (SD) and coefficient of variance (CV, %).

Strains	Mean (SD)	CV
pGAL4	0.093 (± 0.023)	21
pGAL4.VP16	0.040 (± 0.012)	30
pGAL4.*Ci*LBD	0.140 (± 0.015)	11
pGAL4.*Bs*LBD	0.086 (± 0.010)	12
pGAL4.*Ci*LBDΔ31.VP16	0.268 (± 0.013)	5
pGAL4.*Ci*LBD.VP16	0.302 (± 0.013)	4
pGAL4.*Bs*LBD.VP16	0.294 (± 0.016)	5

Due to the size of this study, it was necessary to run samples on multiple assay plates. Intra-assay CVs were calculated to validate these yeast bioassay results for each test compound. Coefficients of variance were <25% for two synthetic chemicals, *n*-butyl-*p*-aminobenzoate and carbamazepine, used as positive controls for the yeast bioassays (Table 3.1). Reliable results for two additional synthetic chemicals, *p*-aminobenzoic acid and BPA, were obtained in yeast strains expressing the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Ci*LBD.VP16; CV <25%). In contrast, induction of β-galactosidase by these compounds was highly variable (CV >85%) in yeast strains expressing the *B. schlosseri* VDR/PXRα LBD (Table 3.1). The yeast bioassays were highly sensitive towards two microalgal biotoxins, portimine and okadaic acid, as indicated by low CV values (CV <25%; Table 3.2). One exception was the yeast strain expressing the truncated version of the *C. intestinalis* VDR/PXRα LBD with β-galactosidase activities being highly variable in response to okadaic acid (CV = 46%; Table 3.2). Induction of β-galactosidase activity in response to PTX-11 was very inconsistent in all three positive yeast strains (CV = 35–46%; Table 3.2).

Based on the assay variability observed for BPA, *p*-aminobenzoic acid and PTX-11, it appears that the yeast bioassay is not sensitive enough to reliably detect these compounds. This may be due to the β-galactosidase concentration responses being very low for BPA, *p*-aminobenzoic acid and PTX-11 (corrected OD_{540} <0.2; Figure 3.6C–D and 3.7B). In comparison, induction of β-galactosidase activity by portimine was at least three-times greater than for PTX-11 (corrected OD_{540} = 0.6–1.5; Figure 3.7B, C) resulting in very low intra-assay variability (CV = 7–12%; Table 3.2).

The observed variability may also be attributed to low or limited solubility of some test compounds in ethanol. Thus, all compounds were dissolved in DMSO (Section 3.2.1). Of the nine compounds tested, three (*n*-butyl-*p*-aminobenzoate, BPA and PTX-11) were active in the yeast bioassays (Appendix 11). Although the yeast bioassays were more sensitive towards *n*-butyl-*p*-aminobenzoate when dissolved in DMSO than in ethanol (EC_{50} = 2.4–9.4 µM; Table 3.1, Appendix 11A), the within-assay variability was greater (CV >25%; Table 3.1, Appendix 11A). The opposite effect was observed for BPA. When dissolved in

DMSO, assay sensitivity decreased by one order of magnitude (EC_{50} = 58–76 µM; Appendix 11A), while intra-assay variability improved (CV <17%; Appendix 11A). It appeared that both solvents have differential effects on assay sensitivity for particular compounds within the concentration ranges tested in this study. This is consistent with a previous study showing that the yeast estrogen receptor (ERα) reporter assay is more sensitive using ethanol as a solvent compared to DMSO (Bovee *et al.* 2004).

The yeast bioassays showed similar levels of low sensitivity and high variability (EC_{50} = 545–913 nM, CV = 35–66%; Table 3.2; Appendix 11B) to PTX-11 dissolved in either ethanol or DMSO. These EC_{50} values are one order of magnitude higher than the EC_{50} value reported for pectenotoxin-2 (PTX-2; EC_{50} = 37 nM) in a mammalian cell line (Fidler *et al.* 2012). It is important to note that two different pectenotoxin analogues were used in the two studies. Pectenotoxin-11, used in this study, differs slightly from PTX-2 in having an additional hydroxyl group at C 34 (Suzuki *et al.* 2006). This may change its affinity for the receptor LBD slightly. Both PTX analogues are well known microalgal biotoxins, sharing the same mode of action which can cause diarrhetic shellfish poisoning in vertebrates (Dominguez *et al.* 2010; Reguera *et al.* 2012), thus being likely ligands of tunicate VDR/PXRs.

A range of additional observations arise from this study that should be considered in developing similar/alternative yeast bioassays based on VDR/PXR orthologues from tunicate species. Yeast strains producing chimeric proteins that have the GAL4-DBD and VDR/PXRα LBD but lacking the VP16-AD showed no ligand-dependent induction of β-galactosidase enzymatic activity. This indicates that the transcription activation domains expected to be present within VDR/PXRα LBDs are not functional in yeast cells (Berry *et al.* 1990; Louvion *et al.* 1993). Therefore, any similar/alternative yeast bioassays developed in future should include a C-terminal VP16-AD in the chimeric proteins designed. The differing responses of yeast strains expressing *C. intestinalis* VDR/PXRα LBDs of varying length highlights a need to consider the effect of the expressed LBD region on the yeast bioassay responses to test compounds. In general, it appeared that for VDR/PXR proteins it is important to

include all sequences C-terminal to the predicted DBD rather than relying upon computer programs to delimit the N- and C-terminal boundaries of the LBD.

3.4 Conclusion

Recombinant yeast strains were generated that express two orthologue tunicate VDR/PXRα LBDs fused, at their N-termini, with the generic GAL4-DBD and, at their C-termini, with a generic transcription activation domain (VP16-AD). These chimeric proteins were expressed in a yeast host strain, which encodes a *lacZ* reporter gene adjacent a GAL4-controlled promoter. The resulting strains increase β-galactosidase enzymatic activity in a concentration-dependent manner in the presence of putative VDR/PXRα LBD ligands. This effect was not found in strains transformed with plasmids encoding proteins with the GAL4-DBD and VP16-AD but lacking a VDR/PXRα LBD, proving that the β-galactosidase induction effect was mediated through the tunicate VDR/PXRα LBDs.

These yeast bioassays proved useful for the detection of both synthetic chemicals and natural microalgal biotoxins. The yeast bioassay was highly reliable for repeated detection of a well-known *C. intestinalis* VDR/PXRα LBD agonist, *n*-butyl-*p*-aminobenzoate (Ekins *et al.* 2008). However, assay sensitivity varied depending on the type of compounds tested and the organic solvent used. These data indicated that both induction of β-galactosidase enzymatic activity (measured as corrected OD_{540} values) greater than 0.2 and sigmoidal response curves are important for achieving trustworthy results (CVs <25%). Future research should therefore aim to focus on these criteria in order to improve assay sensitivity and reliability. This may be achieved by first determining the ideal solvent for a particular compound before being tested in the yeast bioassay (Bovee *et al.* 2004). The percentage of the solvent used in the final bioassay may also influence sensitivity (Bovee *et al.* 2004). It may, in some cases, be better to use DMSO because this solvent does not evaporate as quickly as ethanol, which may minimise assay variability.

The β-galactosidase enzymatic activity induction curves that were observed were found to be dependent on the histidine concentration in the yeast bioassay media. This indicates that induction of β-galactosidase activity is the actual sum of activation of two GAL4-DBD-activated co-regulated promoters. While this reflects the specific yeast host strain selected, it appears to result in clearer ligand-dependent response curves suggesting that the specific host strain used in this work has inherent advantages for GAL4-DBD-based yeast bioassays.

Despite the success of the yeast bioassays for some test chemicals described in this chapter, it is important to be aware of a limitation common to all cell-based assays. The test chemicals must cross the cell membrane, either passively or by active transport. They also must be available within the cell cytoplasm in a form that can interact with the chimeric fusion protein (Norcliffe *et al.* 2013). In addition, yeast cells possess specific mechanisms for the elimination of exogenous toxic compounds, which may reduce the sensitivity of the yeast bioassays to such chemicals (Norcliffe *et al.* 2013). While such considerations do not undermine the usefulness and validity of these yeast bioassays, they may limit the range of chemicals for which bioactivity can be detected.

3.5 References

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Sampling of *Botryllus schlosseri* colonies was undertaken from Michmoret beach by the Ruppin Academic Centre, Mediterranean Coast, Israel.

CHAPTER FOUR

Utilising tunicate xenobiotic receptors in yeast bioassays for high-throughput screening of bioactive compounds

4.1 Introduction

The development of robust high-throughput screening (HTS) assays for natural marine bioactive compounds for pharmaceutical purposes is technically challenging (Martins *et al.* 2014). Consequently, despite the enormous number of structurally unique bioactive marine natural products that are known, there are only eight approved drugs, along with 12 natural marine products (or derivatives thereof) in different phases of clinical testing (Mayer *et al.* 2010; Martins *et al.* 2014). The development of better bioassays is a key factor for identifying the activity of bioactive chemicals, because the natural biological activities of putative drug compounds influence their potential medical applications (Imhoff *et al.* 2011; Martins *et al.* 2014).

A number of nuclear receptors (NRs) have been used successfully during the early phases of drug discovery (Shi 2007). Among the HTS assays, ligand-binding and cell-based NR transactivation assays (Raucy & Lasker 2010, 2013) are of particular importance in drug discovery and are being used by a growing number of pharmaceutical companies (Chu *et al.* 2009; Fahmi & Ripp 2010). Most of the assays are based on mammalian-derived cell lines and screen for compounds that bind to and/or activate pregnane X receptor (PXR), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR; Michelini *et al.* 2010; Raucy & Lasker 2013). For example, cell-based (HepG2) transactivation luciferase reporter gene assays have been developed to identify both PXR ligands and cytochrome P450 enzyme (e.g. CYP3A4) inducers (Herbst *et al.* 2009). These assays can be used to identify novel drugs and natural products

that modulate PXR activity which may aid the prediction of drug–drug interactions and xenobiotic-induced toxicities (Chang & Waxman 2005).

Despite these applications, mammalian cell lines have significant limitations as they require costly and highly specialised culturing facilities and personnel with advanced laboratory skills (Balaguer *et al.* 1999). The typical mammalian cell line assays that are used (e.g. MCF-7, HeLa) require several days' of growth in steroid-free serum and 16-hour incubations with test chemicals (Balaguer *et al.* 1999). In contrast, baker's yeast (*Saccharomyces cerevisiae*) is a well-established eukaryotic expression system that is robust, cost-effective and generally easier/faster to perform than mammalian cell-based assays (Leskinen *et al.* 2005; Balsiger *et al.* 2010). Additionally, yeast bioassays can be adapted to microplate formats and automated making them particularly well-suited for HTS (de Almeida *et al.* 2008).

In Chapter Three, I reported the development of a generic recombinant yeast bioassay capable of detecting marine microalgal biotoxins. These recombinant yeast strains expressed chimeric proteins containing the ligand-binding domains (LBDs) of two tunicate (*Ciona intestinalis* and *Botryllus schlosseri*) genes that are orthologues to the vitamin D receptor (VDR) and PXR (VDR/PXRα). These chimeric proteins mediate ligand-dependent transcription of a reporter gene (*lacZ*) encoding the easily assayed enzyme β-galactosidase. The aim of this chapter was to identify if tunicate VDR/PXR LBDs were activated by marine bioactive compounds, or their chemically modified analogues. It was hoped that this detection system might be useful for detecting new candidates for drug development. Since many drugs used currently are derived from terrestrial organisms, a range of fungal metabolites and one plant-derived natural product were also tested for their activity in the yeast bioassay.

4.2 Materials and methods

4.2.1 Compounds tested in the yeast bioassay

The compounds tested in the yeast bioassay are given in Table 4.1 and their structures in Figure 4.1. The following natural products and their analogues were provided by Prof Margaret Brimble (University of Auckland): gymnodimine, gymnodimine-brominated, gymnodimine-dansylated, gymnodimine-oxidised, paecilospirone, CJ-13-015, CJ-13-102, CJ-13-103, CJ-13-104, CJ-13-108, spirolaxine, spirolaxine-methyl ether, thysanone, (-)-deoxydihydrokalafungin and (+)-deoxykalafungin. Naringin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All chemicals were dissolved in analytical grade ethanol (Merck, Whitehouse Station, NJ, U.S.A.) to form stock solutions. Serial dilutions were added to the yeast bioassay media at a final ethanol concentration of 1% (v/v).

4.2.2 Bioassay procedure

The tunicate VDR/PXRα LBD-based yeast bioassays, data analyses and development of recombinant yeast strains were performed as described in Chapter Three (Section 3.2.2–3.2.7).

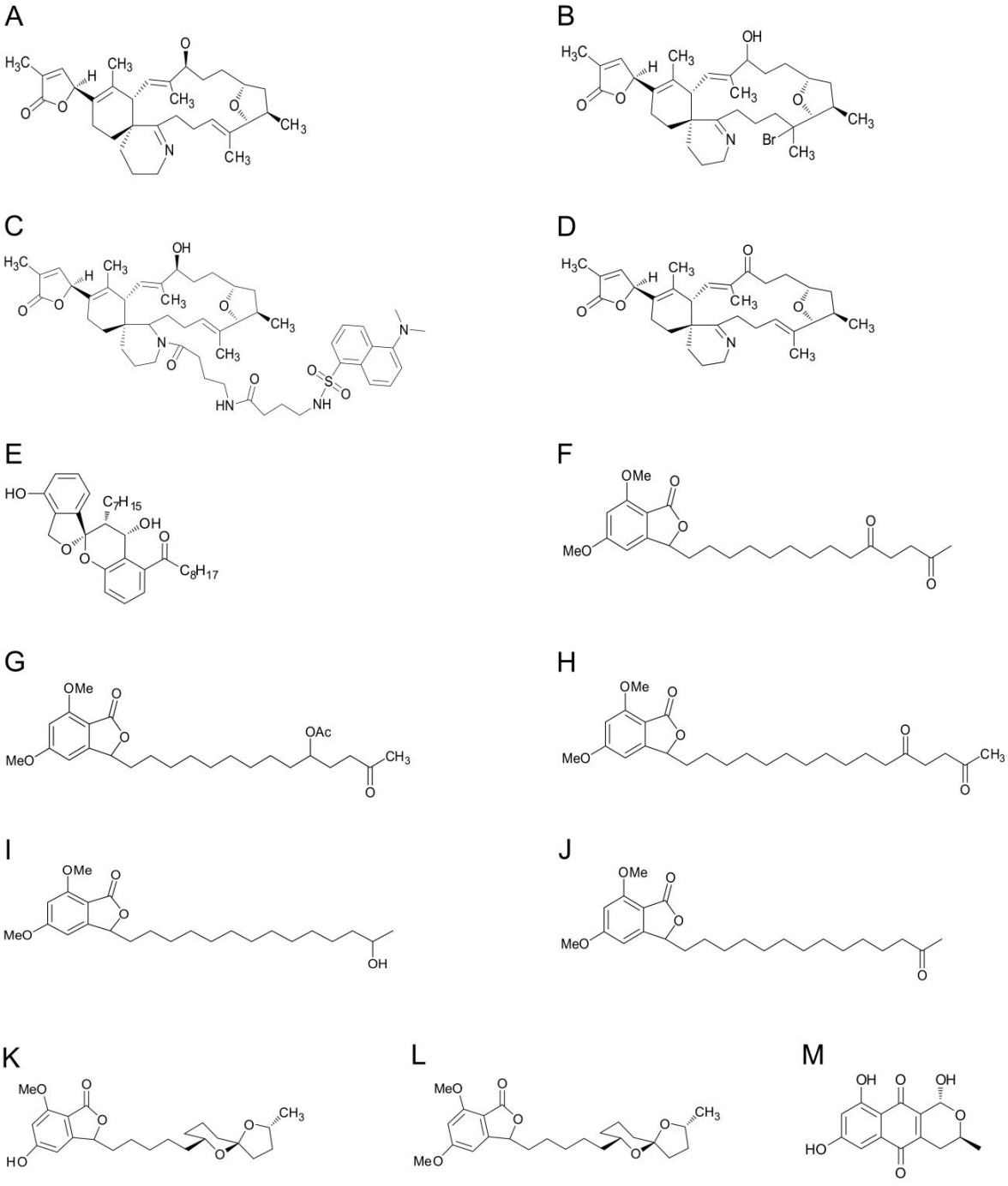

Figure 4.1 Structures of 16 natural products and relevant analogues.

The following chemicals were tested in tunicate VDR/PXRα LBD-based yeast bioassays: (**A**) gymnodimine, (**B**) gymnodimine-brominated, (**C**) gymnodimine-dansylated, (**D**) gymnodimine-oxidised, (**E**) paecilospirone, (**F**) CJ-13-015, (**G**) CJ-13-102, (**H**) CJ-13-103, (**I**) CJ-13-104, (**J**) CJ-13-108, (**K**) spirolaxine, (**L**) spirolaxine-methyl ether and (**M**) thysanone.

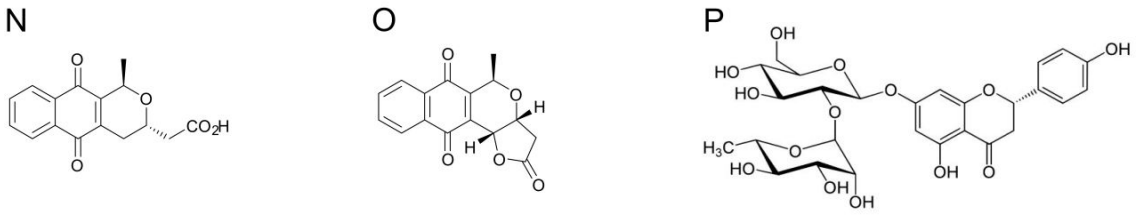

Figure 4.1 (cont.) Structures of 16 natural products and relevant analogues.

The following chemicals were tested in tunicate VDR/PXRα LBD-based yeast bioassays: (**N**) (-)-deoxydihydrokalafungin, (**O**) (+)-deoxykalafungin and (**P**) naringin.

4.3 Results and discussion

The aim of this study was to test if tunicate VDR/PXRα LBD-based yeast bioassays were activated by a range of structurally diverse natural bioactive compounds sourced from both marine and terrestrial organisms. Of the 16 compounds tested, 12 activated both the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD-based yeast bioassays generating EC_{50} values in the low µM range. A plant-derived natural product, naringin, was particularly potent (EC_{50} values in the nM range), while three fungal metabolites and one microalagal biotoxin were inactive (Table 4.1).

Table 4.1 Activation of the tunicate yeast bioassays by 16 natural products and relevant analogues.
Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in μM. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in ethanol.

Chemical name	**Toxin type**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Gymnodimine [b]	Oral toxicity to mice	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Gymnodimine-brominated [b]	Oral toxicity to mice	4.8 (2.4–9.5)	26	4.6 (3.5–6.0)	25	3.3 (2.9–3.7)	22
Gymnodimine-dansylated [b]	Oral toxicity to mice	2.1 (1.9–2.2)	18	2.0 (1.9–2.1)	13	1.9 (1.9–2.1)v	35
Gymnodimine-oxidised [b]	Oral toxicity to mice	6.1 (2.8–13)	37	6.2 (3.4–11)	22	3.8 (2.0–7.1)	21
Paecilospirone [b]	Anti-cancer activities	55 (18–170)	3	15 (3.9–55)	4	~58 (DNC)	46
CJ-13-015 [b]	Potent antibacterial	6.1 (3.7–10)	23	3.7 (2.7–4.9)	24	3.0 (1.7–5.2)	21
CJ-13-102 [b]	Potent antibacterial	~6.6 (DNC)	425	4.7 (3.6–6.2)	12	2.4 (0.4–13)	85
CJ-13-103 [a]	Potent antibacterial	8.5 (5.7–12)	23	15 (9.0–25)	35	2.1 (0.1–42)	40
CJ-13-104 [b]	Potent antibacterial	8.5 (2.9–24)	8	16 (7.6–33)	10	20 (4.7–82)	6
CJ-13-108 [b]	Potent antibacterial	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Spirolaxine [a]	Potent antibacterial	~17 (DNC)	54	18 (14–23)	24	3.3 (0.3–35)	48

Table 4.1 (cont.) Activation of the tunicate yeast bioassays by 16 natural products and relevant analogues.

Chemical name	**Toxin type**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Spirolaxine-methyl ether [a]	Potent antibacterial	33 (23–48)	40	16 (0.5–475)	55	21 (15–28)	65
Thysanone [b]	3C protease inhibitor	3.3 (2.6–4.1)	13	3.0 (2.6–3.4)	4	2.1 (1.7–2.6)	5
(-)-Deoxydihydrokalafungin [a]	3C protease inhibitor	9.7 (8.3–11)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
(+)-Deoxykalafungin [a]	3C protease inhibitor	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Naringin [b]	CYP inhibitor	108 (56–208) [c]	25	67 (47–93) [c]	9	140 (92–211) [c]	25

Abbreviations: DNC, did not compute; NI, no induction of β-galactosidase enzymatic activity; CYP, cytochrome P450.

[a] Strains were incubated for 24 hours.

[b] Strains were incubated for 48 hours.

[c] EC_{50} and 95% CI values for naringin are given in nM.

4.3.1 Activity of microalgal biotoxins in the yeast bioassays

Gymnodimine, produced by the dinoflagellate *Karenia selliformis*, did not induce β-galactosidase activity in any of the yeast bioassays (Table 4.1, Figure 4.2A). The inactivity of gymnodimine is consistent with a study showing inactivity of gymnodimine in mammalian cell lines expressing the *C. intestinalis* VDR/PXRα LBD (Fidler *et al.* 2012). The chemically similar gymnodimine analogues (gymnodimine-brominated, -dansylated and -oxidised) induced a response in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 (Table 4.1, Figure 4.2B–D) suggesting that structural changes alter activation of the tunicate VDR/PXRα LBD. The coefficients of variance (CV) for these four compounds ranged from 13–37% (Table 4.1). The increased assay variability (CVs >25%) can be attributed to shallow concentration-dependent response curves as indicated by the β-galactosidase plateau levels (Figure 4.2B–D).

The response of tunicate VDR/PXRα LBD-based yeast bioassays to different microalgal biotoxins may be due to changes in the physicochemical properties of these compounds. The dansylated gymnodimine analogue, which carries a large dansyl amide side chain (Figure 4.1C), was more potent than the oxidised- and brominated-gymnodimine analogues as indicated by the plateau levels of β-galactosidase production (Figure 4.2B–D). Previous pharmacophore modelling indicated that there were differences in hydrophobicity, molecular weight and the number of hydrogen bond acceptors/donors between microalgal biotoxins that activate the *C. intestinalis* VDR/PXRα LBD (e.g. pectenotoxin-2 and okadaic acid) and those that are inactive (e.g. gymnodimine and yessotoxin; Fidler *et al*. 2012).

The differential activation of the yeast bioassay by the four gymnodimine analogues is in contrast to a previous study reporting that gymnodimine and its chemically modified analogues (gymnodimine-acetate, gymnodimine-methyl carbonate and gymnodamine) have the same effect on cellular viability of Neuro2a neuroblastoma cell lines (Dragunow *et al.* 2005). This difference may be attributed to the different mechanisms the bioassays use to measure a response. While a lethal endpoint is measured in the Neuro2a neuroblastoma

cell line assay, the tunicate VDR/PXRα LBD yeast bioassay measures PXR-ligand interaction through a physiological process. The inactivity of gymnodimine in the yeast bioassay may be explained by the inability of gymnodimine to bind to PXR orthologues in general, since it was also inactive in mammalian cell line assays expressing human PXR, zebrafish PXR, *C. intestinalis* FXR and *C. intestinalis* LXR (Fidler *et al.* 2012). This highlights the potential of using PXR LBD-based yeast bioassays, such as the tunicate VDR/PXRα yeast bioassay, to detect bioactive compounds that represent precursors (e.g. gymnodimine analogues) of the active toxin (e.g. gymnodimine; Dragunow *et al*. 2005).

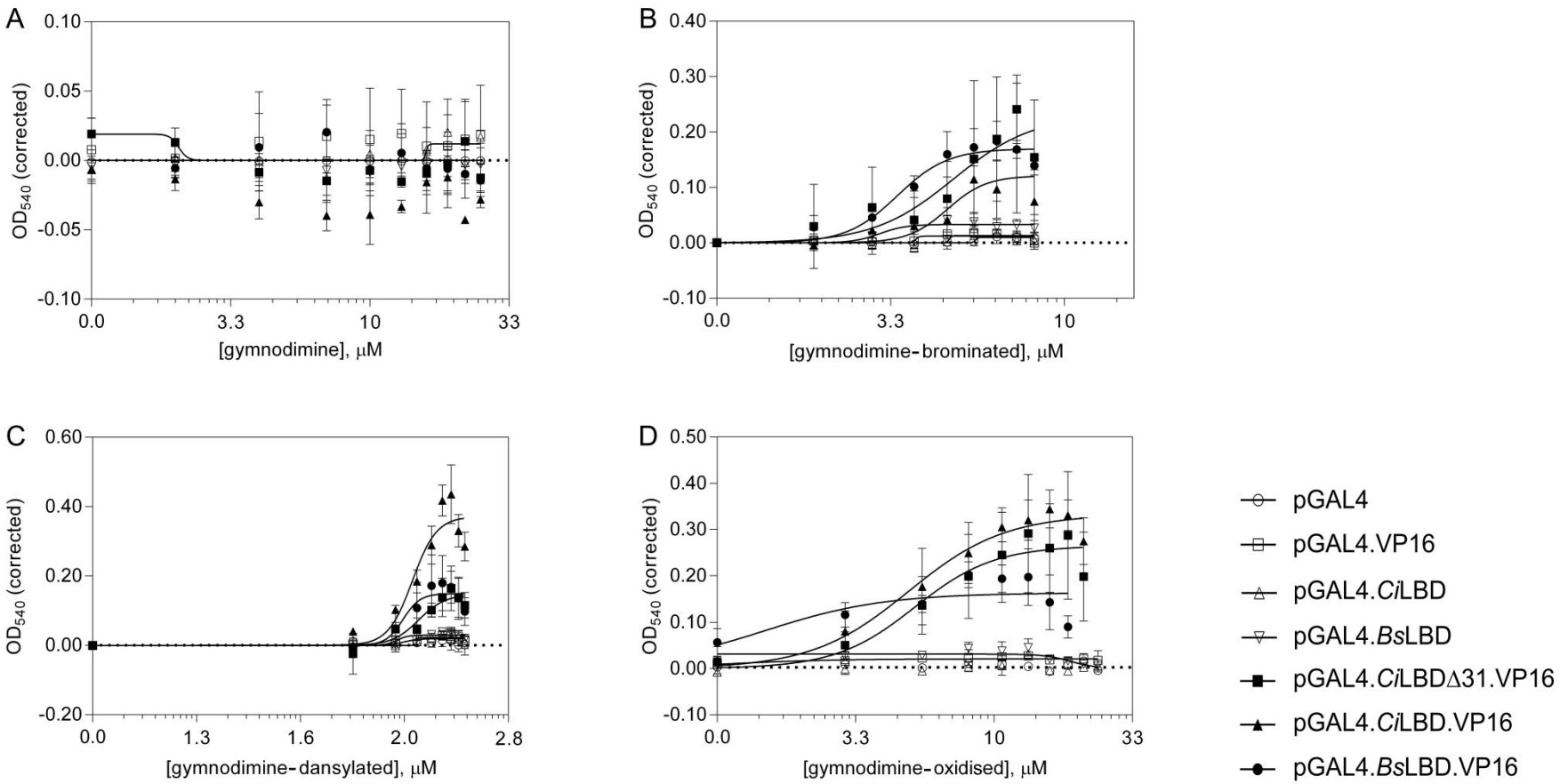

Figure 4.2 Activities of gymnodimine and related analogues tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) gymnodimine, (**B**) gymnodimine-brominated, (**C**) gymnodimine-dansylated and (**D**) gymnodimine-oxidised. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h (Table 4.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

4.3.2 Activity of fungal metabolites in the yeast bioassays

The marine fungal metabolite, paecilospirone, was tested in the yeast bioassay to assess whether tunicate VDR/PXRα LBDs were activated by marine natural products other than microalgal biotoxins. Paecilospirone, a [5,6]-bisbenzannulated spiroacetal, was first isolated from a marine-derived fungus (*Paecilomyces* sp.) collected in tropical and sub-tropical coral reef environments (Namikoshi *et al.* 2000a, b). The yeast strain carrying plasmid pGAL4.*Ci*LBD.VP16 showed sigmoidal concentration-dependent β-galactosidase activity, while responses for strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Bs*LBD.VP16 were non-sigmoidal (Figure 4.3A). The strain expressing the *C. intestinalis* VDR/PXRα LBD, pGAL4.*Ci*LBD.VP16, was more strongly activated by paecilospirone than the strain expressing the *B. schlosseri* VDR/PXRα LBD which showed high variability (Table 4.1, Figure 4.3A). Since paecilospirone is a promising lead compound for the development of chemotherapeutic drugs due to its anti-cancer properties (Sun *et al.* 2011), these data indicate that tunicate VDR/PXRα LBDs could be used as sensor elements in yeast bioassays for identifying other compounds with similar activities.

In addition to marine fungal metabolites, natural products derived from terrestrial fungi have been an excellent source of pharmaceuticals, for example antibacterial penicillin, cholesterol-lowering lovastatin, antifungal echinocandin B and immunosuppressive cyclosporin A (Evidente *et al.* 2014). A small group of bioactive fungal metabolites (CJ-13-015, CJ-13-102, CJ-13-103, CJ-13-104, CJ-13-108, spirolaxine and spirolaxine-methyl ether) has been examined previously for their inhibitory activity against the microaerophilic Gram-negative bacterium *Helicobacter pylori* (Dekker *et al.* 1997; Radcliff *et al.* 2008). Over 50% of the human population are infected by *H. pylori*, which has been associated with increased risk of developing gastric cancer (Montecucco & de Bernard 2003). Four CJ-13 analogues (CJ-13-015, -102, -103 and -104) activated the tunicate VDR/PXRα LBDs (Table 4.1, Figure 4.3B–E) suggesting that the yeast bioassay could be useful for identifying other compounds with antibacterial activity. The *B. schlosseri* VDR/PXRα LBD-based yeast bioassay

(pGAL4.*Bs*LBD.VP16) was less sensitive than the *C. intestinalis* VDR/PXRα equivalent as indicated by the plateau levels of β-galactosidase production (Figure 4.3D–E). However, this difference was not reflected in the EC_{50} values (Table 4.1). In contrast, CJ-13-108, was inactive in the yeast bioassay (Table 4.1, Figure 4.3F), which may be attributed to its limited solubility in organic solvents (Radcliff *et al.* 2008).

Yeast strains expressing the full-length LBD of the *C. intestinalis* and *B. schlosseri* VDR/PXRα receptors were activated by spirolaxine, while the response of yeast strains expressing the truncated *C. intestinalis* receptor (pGAL4.*Ci*LBDΔ31.VP16) were highly variable (Table 4.1, Figure 4.3G). The chemically similar spirolaxine-methyl ether gave positive responses in all three yeast strains, although they were non-sigmoidal and the 95% confidence interval (95% CI) for strain pGAL4.*Ci*LBD.VP16 was very wide (Table 4.1, Figure 4.3H).

Yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16, or pGAL4.*Bs*LBD.VP16 showed sigmoidal concentration-dependent response curves when tested with the clinically important fungal metabolite, thysanone (Table 4.1, Figure 4.3I). The *B. schlosseri* VDR/PXRα LBD-based yeast bioassay (pGAL4.*Bs*LBD.VP16) was less sensitive than the two *C. intestinalis* VDR/PXRα LBD-based yeast bioassays (pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Ci*LBD.VP16; Figure 4.3I). This effect was not reflected in EC_{50} values (EC_{50} = 2.1–3.3 μM; Table 4.1) and these were lower than those reported for the inhibition of the human rhinovirus (HRV) 3C protease in a small peptide screening assay (IC_{50} = 47 μM; Singh *et al*. 1991). Screening and identification of lead structures as therapeutic agents against HRVs represents an important area of future research, because HRVs are responsible for causing 50–80% of common colds in humans (Simancas-Racines *et al.* 2013).

Both (+)-deoxykalafungin and (-)-deoxydihydrokalafungin, chemical analogues of the closely related fungal natural products kalafungin and dihydrokalafungin, showed almost no ligand-dependent induction of β-galactosidase activity in the tunicate yeast bioassays (Figure 4.3J–K). One exception was a strain expressing the shorter version of the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16) that generated an EC_{50} value of 9.7 μM. However,

concentration-response curves indicated reduced β-galactosidase enzymatic activity at higher concentrations of both (-)-deoxydihydrokalafungin and (+)-deoxykalafungin (Figure 4.3J–K). This suppression effect appeared to be an antagonistic effect mediated through the tunicate VDR/PXRα LBD as it is apparent in the other two test strains carrying plasmids pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 and to a lesser degree in negative control strains (Figure 4.3J). The lack of a response from (+)-deoxykalafungin and (-)-deoxydihydrokalafungin could be due to their general toxicity to yeast cells. However, the OD_{620} values from the yeast bioassays did not support this hypothesis for (-)-deoxydihydrokalafungin (Appendix 15A). In contrast, suppression observed at high concentrations of (+)-deoxykalafungin (Figure 4.3K) may be toxicity induced effects as the OD_{620} values were lower (Appendix 15B).

A fluorescence resonance energy transfer (FRET) bioassay found that deoxykalafungin inhibited protein kinase B (AKT), a member of the protein kinase A, G and C families (Korwar *et al.* 2014). The reported IC_{50} value (0.28 μM) from the FRET bioassay is up to two orders of magnitude lower than the EC_{50} values obtained in the yeast bioassay for the range of fungal natural product analogues tested (1.9–33 μM; Table 4.1). Thus, it appears that the yeast bioassay is less sensitive at detecting some compounds when compared with bioassays that do not require reporter cell lines (De *et al.* 2005). This limitation is intrinsic to all assays relying on living cells (and is a major weakness of cell-based bioassays) since test compounds need to cross the cell wall and/or membrane (Lyttle *et al.* 1992).

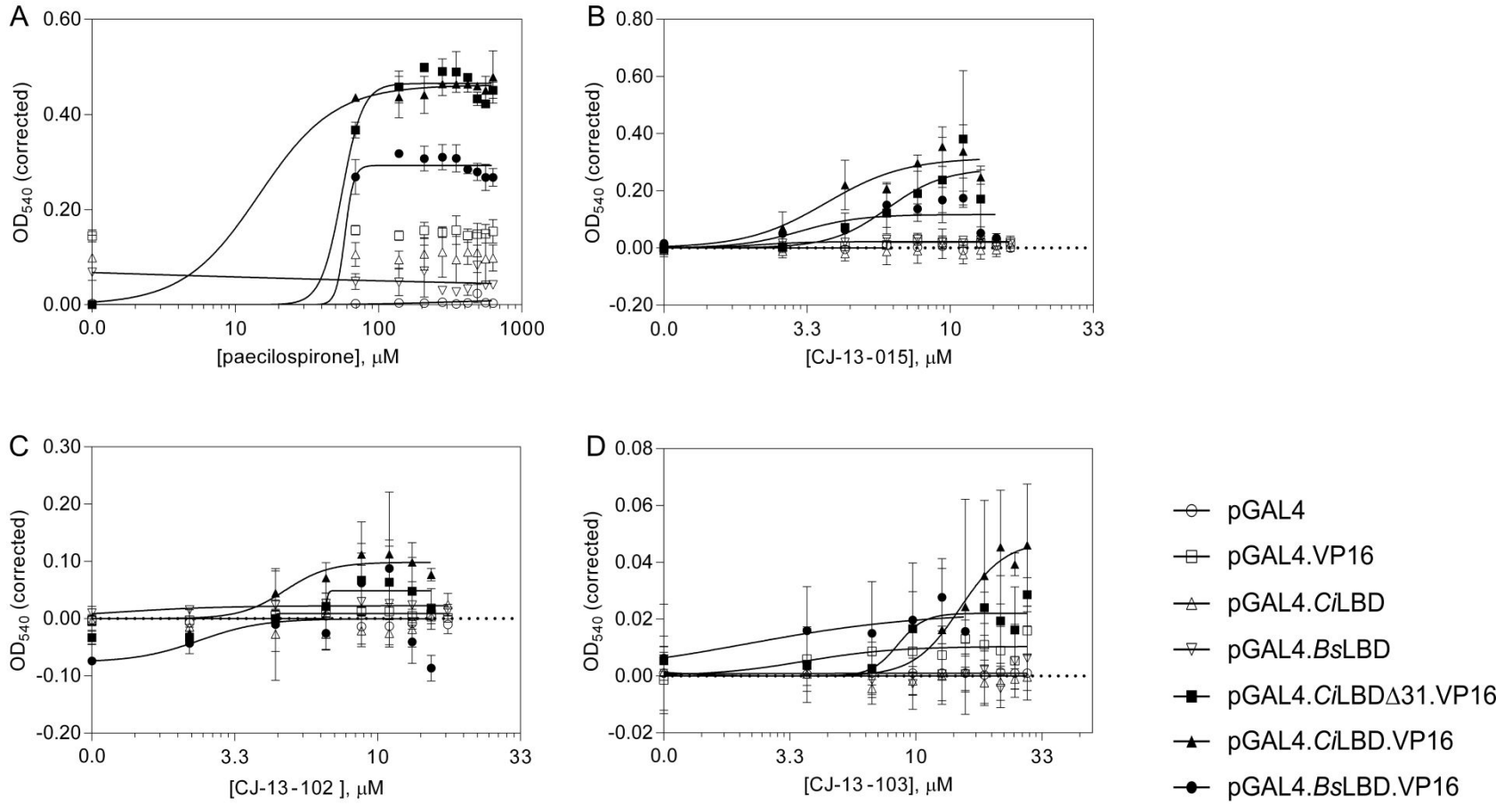

Figure 4.3 Activities of fungal metabolites tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) paecilospirone, (**B**) CJ-13-015, (**C**) CJ-13-102 and (**D**) CJ-13-103. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 24 h or 48 h (Table 4.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

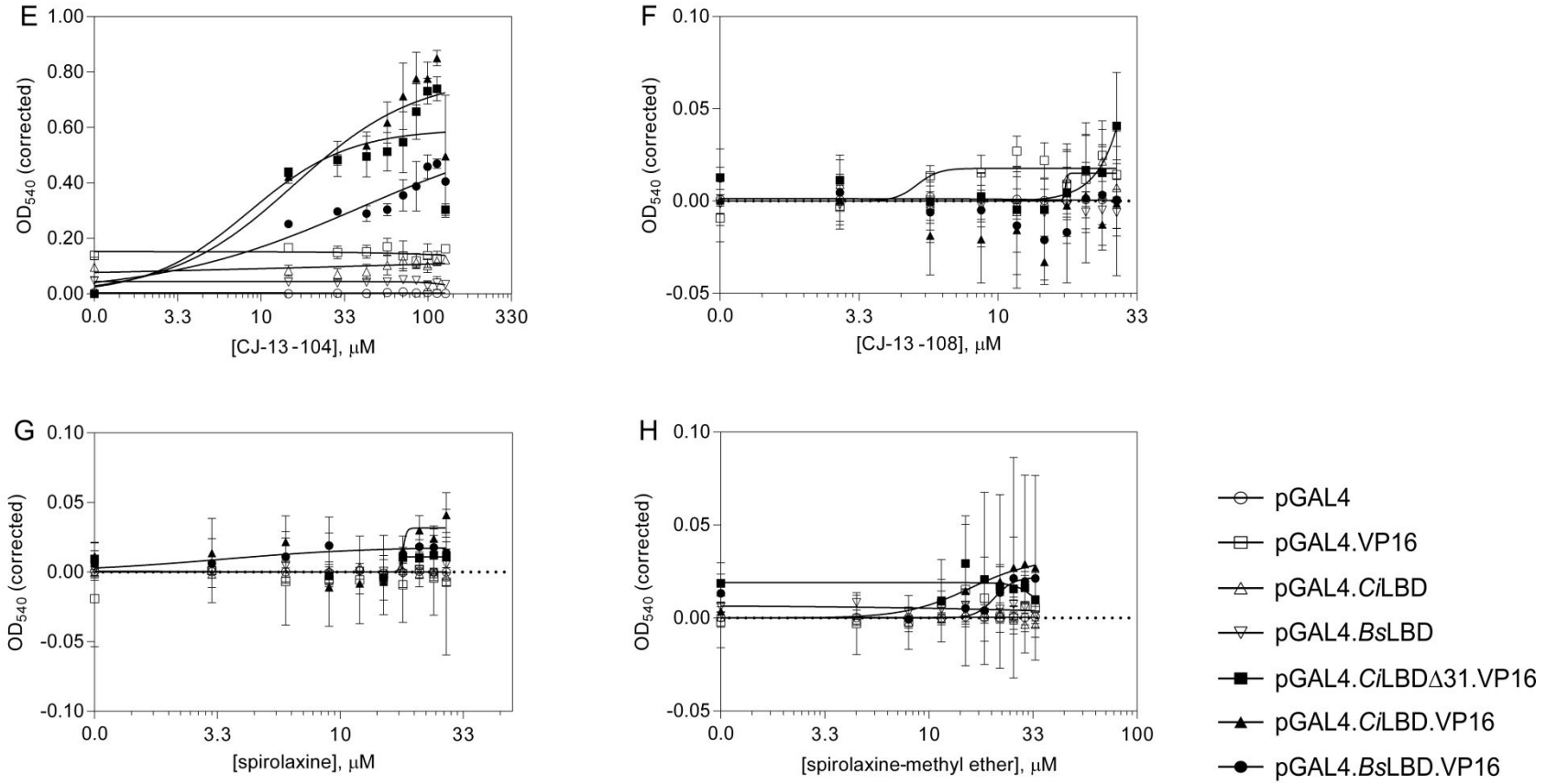

Figure 4.3 (cont.) Activities of fungal metabolites tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**E**) CJ-13-104, (**F**) CJ-13-108, (**G**) spirolaxine and (**H**) spirolaxine-methyl ether.

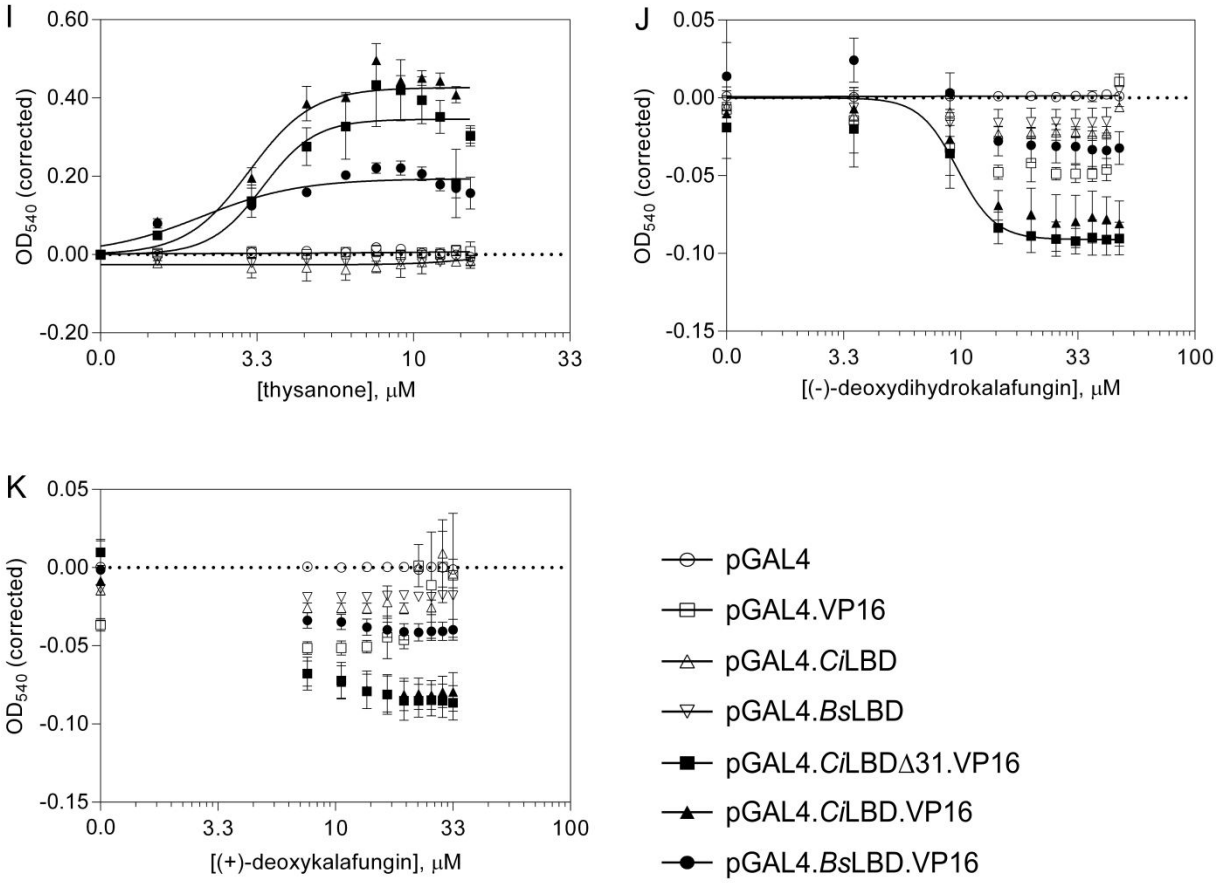

Figure 4.3 (cont.) Activities of fungal metabolites tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of (**I**) thysanone, (**J**) (-)-deoxydihydrokalafungin and (**K**) (+)-deoxykalafungin.

4.3.3 Activity of plant-derived bioactive compounds in the yeast bioassays

Terrestrial plants produce a plethora of bioactive compounds which are used for drug development (Newman *et al.* 2003; Cragg *et al.* 2005; McChesney *et al.* 2007). The plant-derived flavanone naringin was found to be a potent activator of tunicate VDR/PXRα LBDs expressed in recombinant yeast strains (pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16; Figure 4.4) generating EC_{50} values in the low nM range (Table 4.1). Both *B. schlosseri* and *C. intestinalis* VDR/PXRα LBD-based yeast bioassays showed similar levels of β-galactosidase enzymatic activity, while the shorter version of the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16) showed a lower

response as indicated by the plateau levels of β-galactosidase activity (Figure 4.4). Interestingly, the EC_{50} values indicated that naringin was two to three orders of magnitude more potent than the other bioactives and derivatives that were tested during this study (Table 4.1). This may be due to structural similarities between flavonoids (e.g. naringin; Figure 4.1P) and the common pharmacophore of *C. intestinalis* VDR/PXRα ligands. Both compounds consist of a planar aromatic structure with hydrophobic regions and multiple hydroxyl groups (Hodek *et al.* 2002; Ekins *et al.* 2008; Fidler *et al.* 2012).

Naringin, the major flavonoid naturally occurring in grapefruit juice, is of particular interest since it exhibits inhibitory effects on the activity of a number of xenobiotic metabolising enzymes (e.g. CYP1A2 and CYP3A4) *in vivo* (Fuhr 1998; Moon *et al.* 2006). This inhibitory effect can increase the plasma half-life of many clinically important drugs (Fuhr 1998), which can lead to dangerous drug-drug interactions; such as fatal interactions of azole antifungals with cardiovascular agents (Bates & Yu 2003; Cascorbi 2012). However, the actual inhibitory effect on a wide range of detoxification enzymes (e.g. CYPs) is not mediated through naringin but through its metabolite, naringenin (Selma *et al.* 2009), which was not tested during this study. This highlights a limitation of the yeast bioassay. While the tunicate VDR/PXRα yeast bioassay can detect dietary bioactive compounds that are likely to perturb normal physiological functions through interaction with PXR (Dybdahl *et al.* 2012), metabolic by-products of these compounds cannot be detected. This is an important short-coming of all screening assays. They are limited by the compounds tested in these assays, but cannot be used to detect metabolic by-products which are likely to modulate animal biochemistry and physiology differently (Saad *et al.* 2012).

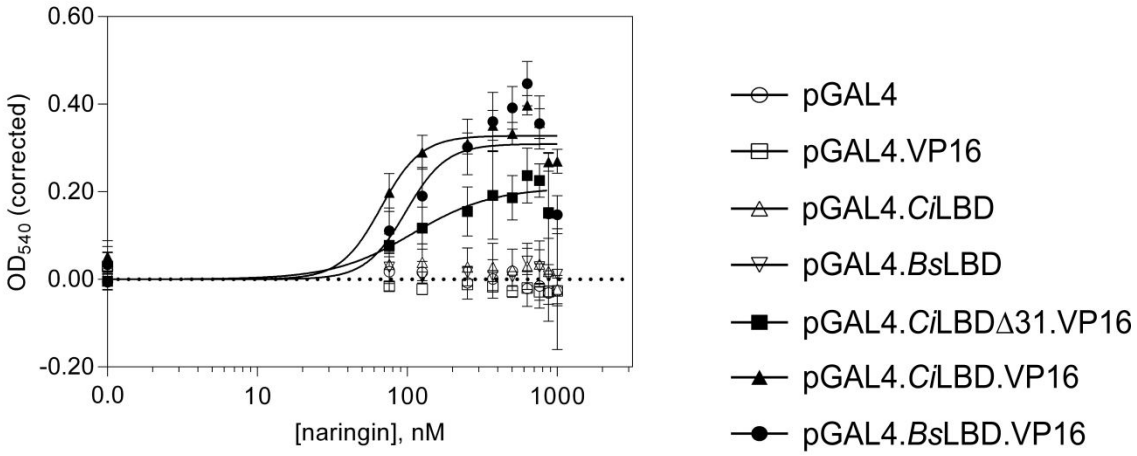

Figure 4.4 Activity of naringin tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of naringin. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h (Table 4.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

4.4 Conclusion

Recombinant yeast strains expressing orthologues of VDR/PXR LBDs from two temperate tunicate species, *C. intestinalis* and *B. schlosseri*, were utilised for the detection of bioactive compounds. These yeast bioassays were activated by a range of marine natural compounds including microalgal biotoxin analogues and fungal metabolites. Terrestrial fungal bioactive compounds and chemically modified analogues were also successfully detected. Since these compounds provide a rich source of drug leads for pharmaceutical purposes (Molinski *et al.* 2009; Imhoff *et al.* 2011), tunicate VDR/PXRα LBDs may represent promising sensor elements for detecting both marine and terrestrial bioactive compounds for drug development. Additionally, the plant-derived flavonoid naringin which can cause dangerous herb-drug interactions in humans (Fuhr 1998) was a potent activator in the tunicate yeast bioassay.

In this study pure compounds were analysed in the yeast bioassays. This highlights the feasibility of using tunicate VDR/PXRα LBD-based yeast bioassays for detecting bioactive compounds from chemical libraries. Future research should aim to identify medically and/or physiologically active compounds from more complex matrices such as chemical extracts or environmental samples.

Further development of the yeast bioassay into a HTS assay format is particularly important, since this is the most widely used approach for the identification of novel drug lead compounds (Hughes *et al.* 2011; Martins *et al.* 2014). The yeast bioassay developed here is particularly well-suited for HTS as it is user-friendly and robust. This is due to the reporter gene *lacZ* which encodes the enzyme β-galactosidase. Enzymatic activity can be measured without the need for cell lysis and requires minimal hands-on time. Additionally, assay miniaturisation, one of the main requirements in HTS development, is possible since the nutrient requirements of yeast are easily met in 96-well plate formats (Wolcke & Ullmann 2001; Hontzeas *et al.* 2007; Norcliffe *et al.* 2013). For example, yeast-based luciferase reporter assays for human estrogens, androgens and xenobiotics have been miniaturised from the standard 96-well plate format to high throughput 384- and 1536-well plate formats (Rajasarkka & Virta 2011).

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Microalgal bloom observed in Cook Strait, New Zealand. 25th October 2009

Image source: National Aeronautics and Space Administration, U.S.A.

CHAPTER FIVE

Utilising tunicate xenobiotic receptors in yeast bioassays for detection of environmental pollutants

5.1 Introduction

Rapid industrial and urban development in the second half of the 20th century has resulted in the emergence of thousands of persistent anthropogenic chemicals (Stewart *et al.* 2014). These include industrial chemical contaminants which are of environmental concern; i.e. heavy metals (especially zinc, copper and lead), polycyclic aromatic hydrocarbons (PAHs) and pesticides (Järup 2003; Karami-Mohajeri & Abdollahi 2011; Ball & Truskewycz 2013). Recently, a new class of non-regulated environmental pollutants, termed emerging contaminants (ECs), has captured scientific and public attention because it may pose a health risk to many organisms, including humans (Gavrilescu *et al.* 2015). Emerging contaminants are continuously released into the aquatic environment through products such as pharmaceuticals, personal-care products and surfactants (Gavrilescu *et al.* 2015) and are considered to be 'pseudo-persistent' (Daughton 2002). Emerging contaminants generally occur in surface and ground waters as mixtures at concentrations which are far below those thought to be harmful (e.g. ng/L to µg/L; Daughton & Ternes 1999). However, the possibility exists that chronic exposures to these low levels may elicit ecotoxicological effects in many taxa (Fent *et al.* 2006; Vasquez *et al.* 2014).

Surfactants are an important class of ECs which have recently been recognised as priority contaminants of concern under the European Commission Water Framework Directive (European Commission 2008). These detergent-like compounds (e.g. dioctyl sodium sulfosuccinate) are the key component of oil dispersants which are used frequently in response to oil spills (Fiocco & Lewis 1999). Although the acute effects of oil dispersants are low compared to crude oil (Fuller *et al.* 2004), the large amounts of dispersants used in response to oil

spills (e.g. approximately 10–100 ppb was used in the Deepwater Horizon oil spill in the Gulf of Mexico) pose significant health risks to a wide range of marine organisms (Kujawinski *et al.* 2011; Wise & Wise 2011; Goodbody-Gringley *et al.* 2013; Almeda *et al.* 2014).

The recent development of integrated bioanalytical approaches using *in vitro* bioassays has become a powerful tool to detect and identify bioactive compounds within complex environmental samples (Eggen & Segner 2003). *In vitro* reporter gene bioassays based on the mechanism of action for chemicals (e.g. hormone receptor activation) are now commonly used as screening tools for sensitive and specific detection of xenobiotic-like activities in complex samples (Kinani *et al.* 2010). To date, most attention has been directed towards identifying endocrine-disrupting chemicals (EDCs), because these compounds can disturb the endocrine system via interaction with steroid hormone receptors (Kojima *et al.* 2010; De Coster & van Larebeke 2012), such as the estrogen (ER) and androgen receptors (Thomas *et al*. 2002; Kinani *et al*. 2010; Colosi & Kney 2011). Other key nuclear receptors (NRs) such as pregnane X receptor (PXR; Timsit & Negishi 2007), peroxisome proliferator-activated receptor (Grun & Blumberg 2009) or glucocorticoid receptor (Odermatt *et al*. 2006), are also known to be directly activated by environmental contaminants, and have potential to be used as xenobiotic sensors for environmental bioanalytical purposes.

Pregnane X receptor is of high interest since it is activated by a diverse range of environmental ligands including steroids, pharmaceutical drugs, pesticides and polybromodiethylethers (Jacobs *et al.* 2005; Lemaire *et al.* 2006; Sinz *et al.* 2006). Reporter gene bioassays, based on cultured HeLa cells that permanently express the luciferase reporter gene under the control of a chimeric human PXR, have been developed for the detection of several pesticides (Lemaire *et al.* 2006), as well as numerous environmental chemicals (i.e. alkylphenols, hormones, pharmaceuticals, pesticides, polychlorinated biphenyls and bisphenol A) in aquatic samples (Creusot *et al.* 2010; Mnif *et al.* 2011; Creusot *et al.* 2013). Due to the extreme number of organic pollutants present in the environment, *in silico* models (e.g. quantitative structure–activity relationship, QSAR) based on human PXR and other NRs have been developed (Vedani *et*

al. 2009; Dybdahl *et al.* 2012). These models are used for initial screening of compounds before selecting a smaller set of potentially harmful candidates for future *in vitro* reporter assays. Similar QSAR *in silico* models are available for various aquatic taxa, although these models are based on experimentally measured *in vivo* toxicities (Agatonovic-Kustrin *et al.* 2014; Golbamaki *et al.* 2014; Singh *et al.* 2014).

The aim of this chapter was to explore the use of the tunicate VDR/PXRα yeast bioassay (Chapter Three) for the detection of anthropogenic chemicals of environmental relevance. This *in vitro* bioassay is based on recombinant yeast (*Saccharomyces cerevisiae*) strains that express chimeric proteins containing the ligand-binding domains (LBDs) of two tunicate PXR orthologues (*Ciona intestinalis* and *Botryllus schlosseri* VDR/PXRα) which mediate ligand-dependent transcription of a reporter gene (*lacZ)*. Thirteen synthetic chemicals were tested in the yeast bioassays. These compounds were selected based on (i) their aromatic, planar structures and hydrophobic features (Ekins *et al.* 2008) and (ii) their classification as ECs (e.g. pesticides, pharmaceutical drugs and preservatives). In addition, five complex chemical mixtures that are frequently used to mitigate marine oil spills were analysed. The tunicate VDR/PXRα yeast bioassay may provide a reliable and inexpensive tool for environmental monitoring of anthropogenic pollutants.

5.2 Materials and methods

5.2.1 Compounds tested in the yeast bioassay

The synthetic compounds tested in the yeast bioassay are given in Table 5.1 and their structures in Figure 5.1. All synthetic compounds were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) with the following exceptions: ketoconazole (EMD Chemicals, Gibbstown, NJ, U.S.A.), 2-phenylphenol (Acros Organics, Geel, Belgium), radicicol (Cayman Chemical, Ann Arbor, MI, U.S.A) and *n*,*n*-diethyl-*m*-toluamide (DEET; Fluka, St. Louis, MO, U.S.A.). All

compounds were dissolved in analytical grade ethanol (Merck, Whitehouse Station, NJ, U.S.A.; Table 5.1) or dimethyl sulfoxide (DMSO; Sigma-Aldrich; Appendix 16) to form stock solutions (Bovee *et al.* 2004). Commercial oil dispersants were sourced from within the Cawthron Institute (Dr Louis Tremblay) and were diluted in ethanol only (Table 5.2). Serial dilutions were added to the yeast bioassay media at a final solvent concentration of 1% (v/v).

5.2.2 Yeast bioassay procedure

The tunicate VDR/PXRα LBD-based yeast bioassays, data analyses and development of recombinant yeast strains were performed as described in Chapter Three (Section 3.2.2–3.2.7).

Based on published EC_{50} values and *in vivo* toxicity data, a wide concentration range (0–1 mM) was selected for initial analyses of test compounds in the bioassays. Each compound was tested at least twice in separate assays. These data were then used to identify a concentration range that induced β-galactosidase enzymatic activity in the bioassay while being non-toxic to the yeast cells themselves. Using the identified concentration range, all yeast bioassays were performed in pseudo-triplicates (triplicate measurements within the same assay). Solvents, ethanol or DMSO (final concentration 1% (v/v); Bovee *et al*. 2004), were tested alongside each test compound in each assay. To facilitate intra-plate comparisons, the coefficient of variance (CV) was calculated for each test compound as described in Chapter Three (Section 3.2.7).

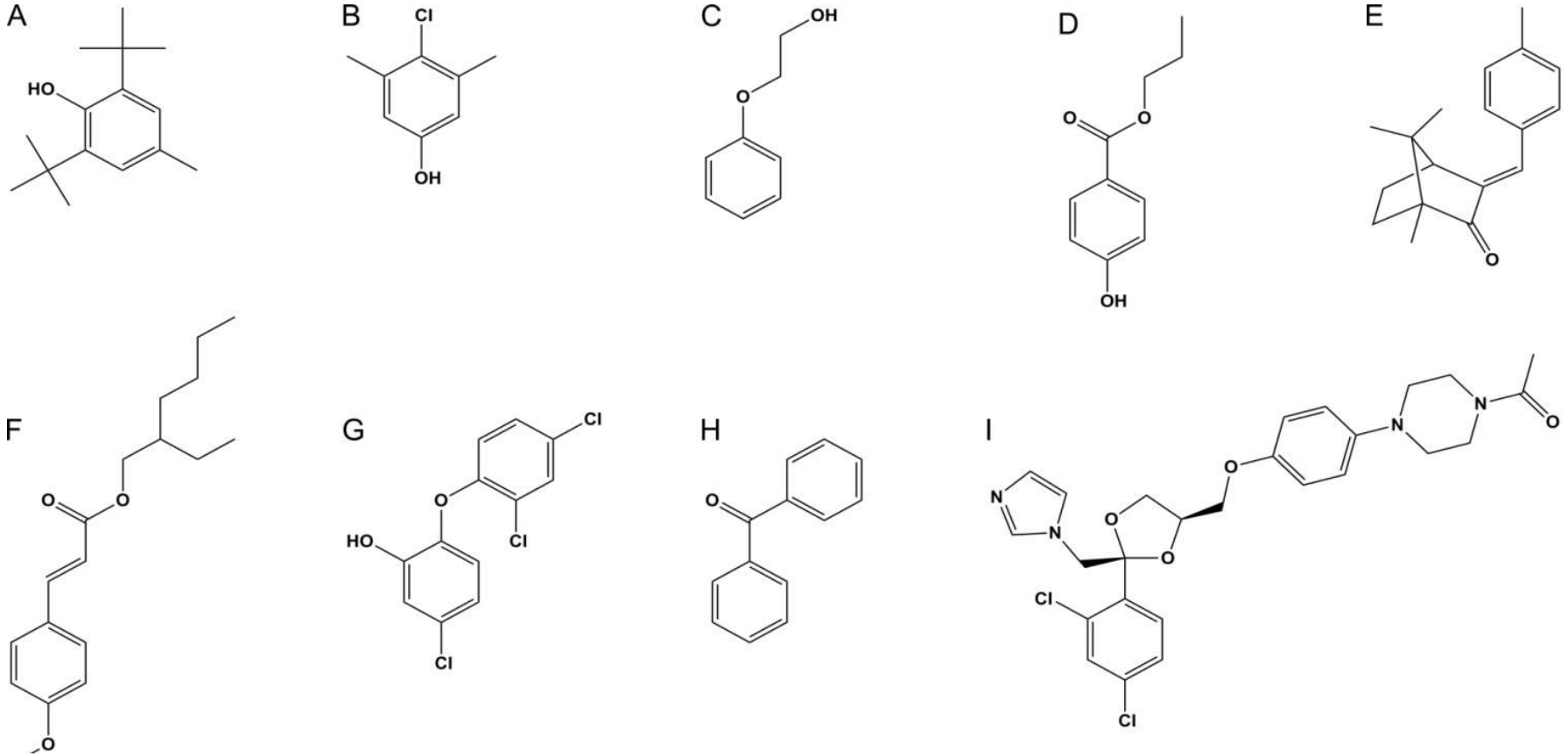

Figure 5.1 Structures of 13 emerging contaminants tested in the yeast bioassays.

The emerging contaminants tested in tunicate VDR/PXRα LBD-based yeast bioassays were: (**A**) butylated hydroxytoluene, (**B**) 4-chloro-3,5-dimethylphenol, (**C**) 2-phenoxyethanol, (**D**) propyl-4-hydroxybenzoate, (**E**) 4-methylbenzylidene camphor, (**F**) octyl methoxycinnamate, (**G**) triclosan, (**H**) benzophenone and (**I**) ketoconazole.

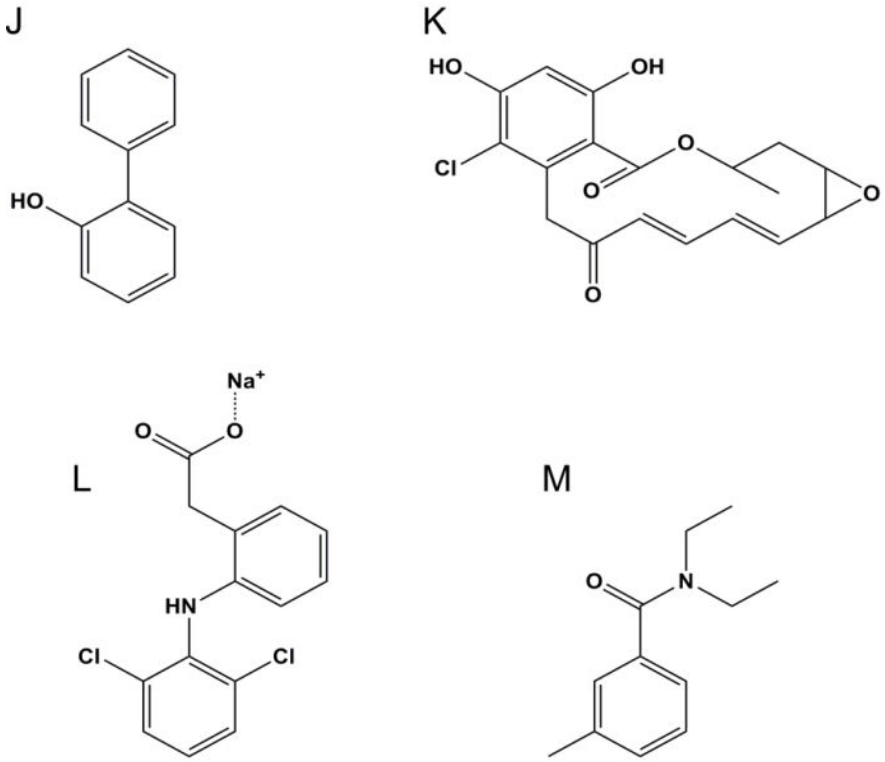

Figure 5.1 (cont.) Structures of 13 emerging contaminants tested in the yeast bioassays.

The emerging contaminants tested in tunicate VDR/PXRα LBD-based yeast bioassays were: (**J**) 2-phenylphenol, (**K**) radicicol, (**L**) diclofenac sodium salt and (**M**) *n*,*n*-diethyl-*m*-toluamide (DEET).

5.3 Results and discussion

5.3.1 Activity of synthetic chemicals in the tunicate yeast bioassays

The first aim of this study was to explore the potential use of the tunicate VDR/PXRα LBD-based yeast bioassay (Chapter Three) for the detection of synthetic chemicals (e.g. pharmaceuticals, pesticides and fungicides) classified as ECs (Figure 5.1). Of the 13 compounds tested, nine activated both the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD-based yeast bioassays generating EC_{50} values in the µM range (Table 5.1).

5.3.1.1 Activity of synthetic preservatives

The four structurally simple synthetic chemicals, 2-phenoxyethanol, 4-chloro-3,5-dimethylphenol, propyl-4-hydroxybenzoate and butylated hydroxytoluene (Figure 5.1A–D), are frequently used as preservatives in foods, drugs and cosmetics (Soni *et al.* 2001; Meyer *et al.* 2007) and these were active in the yeast bioassays (Table 5.1, Figure 5.2A–D). However, only the antioxidant butylated hydroxytoluene exhibited sigmoidal concentration-response curves (Figure 5.2A) which were consistent across assays (CV <20%; Table 5.1). Identification of butylated hydroxytoluene as an activator of both tunicate VDR/PXRα LBDs tested (EC_{50} = 15–30 µM; Table 5.1) was reasonably consistent with its reported *in vivo* toxicity towards the colonial tunicate *B. schlosseri* at concentrations <68 µM (Voskoboynik *et al.* 2002).

Table 5.1 Activation of the tunicate yeast bioassays by 13 emerging contaminants.
Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in µM. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in ethanol.

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Butylated hydroxytoluene [a]	Antioxidant	Sigma-Aldrich (B1378)	30 (21–42)	20	22 (20–24)	4	15 (11–21)	11
4-chloro-3,5-dimethylphenol [b]	Antimicrobial	Sigma-Aldrich (C-4394)	~50 (DNC)	77	62 (26–147)	55	25 (2.2–286)	41
2-phenoxyethanol [b]	Antimicrobial	Sigma-Aldrich (77699)	NI (DNC)	DNC	130 (1.2–13,907)	117	97 (49–193)	154
Propyl-4-hydroxy-benzoate [b]	Antimicrobial	Sigma-Aldrich (P53357)	~388 (DNC)	DNC	NI (NI)	DNC	~979 (DNC)	DNC
4-methyl-benzylidene camphor [b]	EDC	Sigma-Aldrich (78551)	140 (103–189)	33	~125 (DNC)	DNC	107 (84–137)	6
Octyl methoxy-cinnamate [b]	EDC	Sigma-Aldrich (78848)	20 (4.6–87)	30	55 (5.4–561)	51	46 (37–58)	19

Table 5.1 (cont.) Activation of the tunicate yeast bioassays by 13 emerging contaminants.

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Triclosan [a]	EDC	Sigma-Aldrich (72779)	318 (241–422)	35	242 (195–299)	11	NI (DNC)	DNC
Benzophenone [b]	EDC	Sigma-Aldrich (B9300)	221 (194–253)	DNC	63 (38–108)	30	NI (DNC)	DNC
Ketoconazole [a]	Antifungal	EMD Chemicals (420600)	337 (303–375)[c]	5	400 (341–470)[c]	18	~400 (DNC)[c]	DNC
2-phenylphenol [b]	Antifungal	Acros Organics (130760050)	NI (DNC)	DNC	79 (57–112)	37	26 (19–35)	18
Radicicol [b]	Antifungal	Cayman Chemical (13089)	13 (9.1–20)	17	11 (10–12)	9	9.5 (8.5–11)	9
DEET [b]	Insecticide	Fluka (36542)	99 (52–191)	29	54 (32–89)	34	NI (NI)	DNC
Diclofenac sodium salt [b]	Anti-inflammatory	Sigma-Aldrich (06899)	91 (68–121)	41	55 (26–121)	44	11 (0.7–151)	38

Abbreviations: DEET, *n*,*n*-diethyl-*m*-toluamide; DNC, did not compute; EDC, endocrine disrupting chemical; NI, no induction of β-galactosidase enzymatic activity.

[a] Strains were incubated for 24 hours.

[b] Strains were incubated for 48 hours.

[c] EC_{50} and CI values for ketoconazole are given in nM.

Both 2-phenoxyethanol and 4-chloro-3,5-dimethylphenol activated strains carrying plasmids pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 although the responses were non-sigmoidal and inconsistent as indicated by very wide 95% confidence intervals (95% CI) and CVs >25% (Figure 5.2B, C, Table 5.1). It appeared that 2-phenoxyethanol was two orders of magnitude more potent in the tunicate VDR/PXRα LBD-based yeast bioassays (EC_{50} = 97–130 µM; Table 5.1) when compared to the frog (*Xenopus laevis*) embryo teratogenesis bioassay (EC_{50} = 2.5 mM; Vrskova & Modra 2012). However, this comparison is highly tentative due to the considerable variability of β-galactosidase induction by 2-phenoxyethanol (CV = 117–154%). Activation of both tunicate yeast bioassays by 4-chloro-3,5-dimethylphenol (EC_{50} = 25–62 µM; Table 5.1) was also highly variable (CV = 41–554%) but the values were consistent with previous studies which showed that concentrations >50 µM were highly toxic to fish (Houtman *et al.* 2004a).

Paraben (propyl-4-hydroxybenzoate) activated strains carrying plasmids pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Bs*LBD.VP16, but there was high variability (Table 5.1). The concentration-response curves were non-sigmoidal and showed a negative effect on the induction of β-galactosidase activity. This suppression effect was observed in strains carrying the plasmids pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Bs*LBD.VP16, but not in the negative control strains (Figure 5.2D). Examination of OD_{620} values suggests a general toxicity of propyl-4-hydroxybenzoate towards yeast cells (Appendix 17A). In strains carrying plasmids pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Bs*LBD.VP16 this toxic effect was most apparent within a concentration range of 50–350 µM. At concentrations <350 µM the toxic effect decreased which coincided with an increase in β-galactosidase activity at higher concentrations (Figure 5.2D, Appendix 17A). In contrast, a suppression effect was obtained at high concentrations (350–500 µM) in strains carrying plasmid pGAL4.*Ci*LBD∆31.VP16 (Appendix 17A), with these strains showing no ligand-dependent β-galactosidase enzymatic activity (Figure 5.2D). Despite the considerable assay inconsistencies (Table 5.1), the *C. intestinalis* EC_{50} value reported in this study (388 µM) was comparable to those obtained using acute immobilisation assays with *Daphnia magna* (EC_{50} = 127 µM; Terasaki *et al*. 2009).

Collectively, the data from the *in vitro* yeast bioassay and previous *in vivo* studies (Voskoboynik *et al.* 2002; Houtman *et al.* 2004a; Vrskova & Modra 2012) highlight the high toxicity of these synthetic chemicals towards a wide range of aquatic organisms. This is of particular concern since these compounds are being detected with increasing frequency in aquatic environments (Daughton & Ternes 1999; Kolpin *et al.* 2004; Kasprzyk-Hordern *et al.* 2008; Kimura *et al.* 2014). 2-phenoxyethanol can cause serious immunogenic and toxic side-effects in humans (Bis & Mallela 2014), whilst 4-chloro-3,5-dimethylphenol, butylated hydroxytoluene and propyl-4-hydroxybenzoate are practically non-toxic to mammals including humans (Soni *et al.* 2001; Houtman *et al.* 2004a). However, some of these compounds were shown to have weak estrogenic activity when tested in yeast estrogen reporter gene assays and may therefore interrupt normal endocrine functions in animals (Routledge *et al*. 1998; Miller *et al*. 2001).

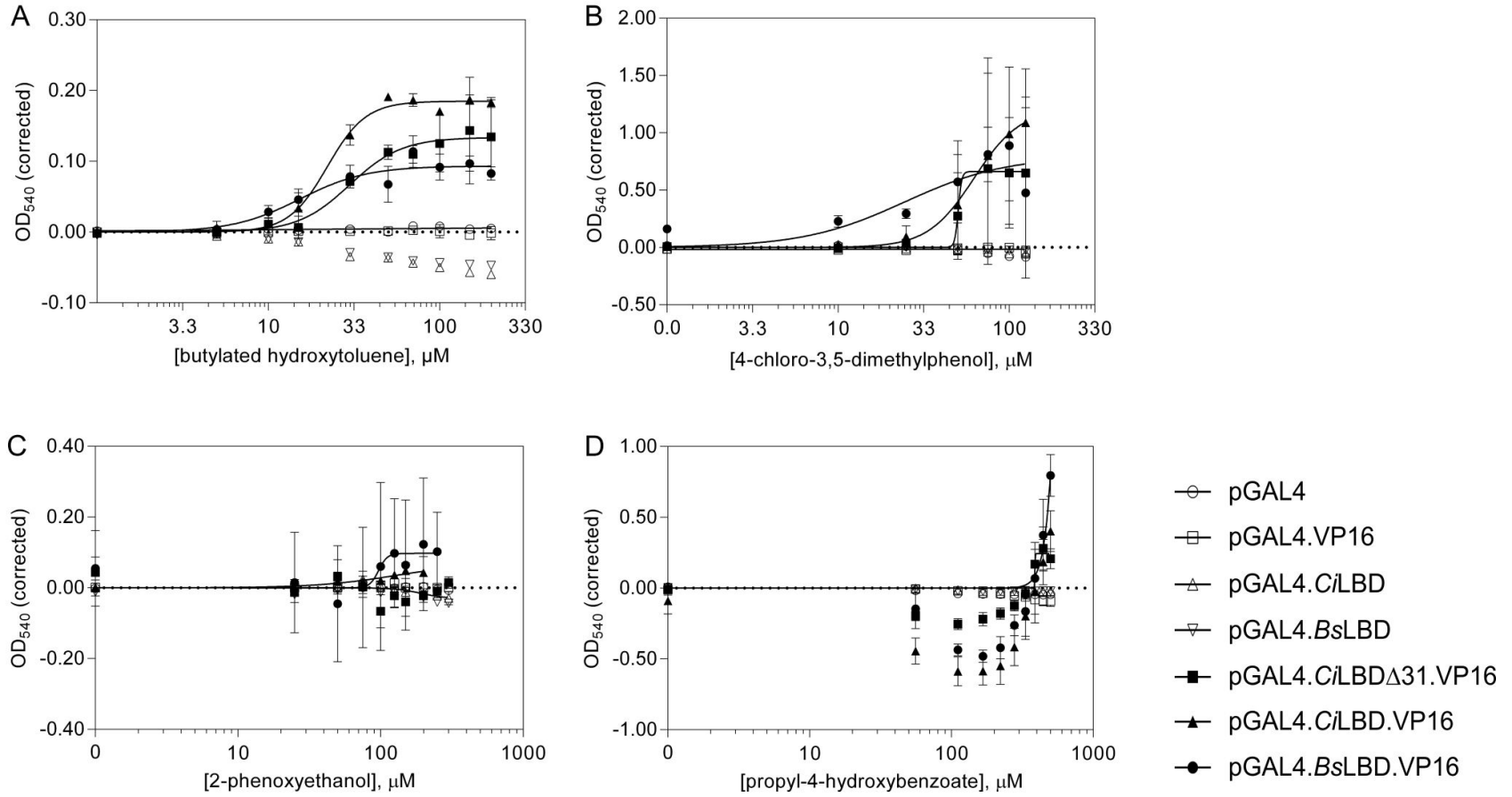

Figure 5.2 Activities of synthetic preservatives tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) butylated hydroxytoluene, (**B**) 4-chloro-3,5-dimethylphenol, (**C**) 2-phenoxyethanol and (**D**) propyl-4-hydroxybenzoate. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 24 h or 48 h (Table 5.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

5.3.1.2 Activity of endocrine disrupting chemicals

Endocrine disrupting chemicals (EDCs) are of particular concern since they can interfere with hormonal regulation and the normal endocrine system (Kojima *et al.* 2010; De Coster & van Larebeke 2012). It is well-known that most EDCs disrupt normal endocrine functions via interaction with sex steroid hormone receptors such as ERα (Routledge & Sumpter 1996; Houtman *et al.* 2004b; Hill *et al.* 2010). Some EDCs may also affect sex steroid hormone receptors indirectly by induction of metabolic enzymes via PXR, leading to disturbance of the endocrine system (Mikamo *et al.* 2003; Jacobs *et al.* 2005).

The two organic ultraviolet absorbing chemicals, 4-methylbenzylidene camphor and octyl methoxycinnamate, tested in this study are used widely in personal care products (e.g. sunscreens) and are known to have estrogenic activity (Miller *et al.* 2001). Both compounds were active in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 (Figure 5.3A–B) although the 95% CIs and CVs of the EC_{50} values were very high (Table 5.1). 4-methylbenzylidene camphor has previously been shown to be highly toxic to *D. magna* using an acute toxicity test, with a reported LC_{50} value of 2.2 µM (Fent *et al.* 2010) being two orders of magnitude lower than the EC_{50} values observed in this study (EC_{50} = 107–140 µM; Table 5.1). Identification of 4-methylbenzylidene camphor and octyl methoxycinnamate as activators of both tunicate VDR/PXRα LBDs (Table 5.1) is consistent with their previously reported anti-estrogenic activities (4-methylbenzylidene camphor, IC_{50} = 87.3 µM; octyl methoxycinnamate, IC_{50} = 4.3 mM) and anti-androgenic activities (4-methylbenzylidene camphor, IC_{50} = 11.8 µM; octyl methoxycinnamate, IC_{50} = 312 µM) using a human ERα yeast transactivation assay (Gomez *et al.* 2005; Kunz & Fent 2006).

Two additional synthetic EDCs (triclosan and benzophenon), with planar structures and at least one hydrogen bond acceptor, were tested in this study (Table 5.1, Figure 5.1G, H). Triclosan was identified as an activator of yeast strains expressing the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Ci*LBD.VP16), although this activation was relatively weak as indicated by the plateau levels of β-galactosidase production (Figure 5.3C). In

strains that express the *B. schlosseri* VDR/PXRα LBD, reduced β-galactosidase enzymatic activity at higher concentrations was observed (Figure 5.3C). This suppression appeared to be an antagonistic effect mediated through the tunicate VDR/PXRα LBD as it was apparent in all strains that carry a tunicate VDR/PXRα LBD but not in any of the negative control strains (Appendix 17B). Activation of tunicate VDR/PXR orthologues by triclosan (EC_{50} = 242–318 µM; Table 5.1) is consistent with that reported by Jacobs *et al.* (2005) who showed that triclosan moderately activates human PXR (46.2% activity relative to the prototypical inducer rifampicin) when transiently transfected into HuH7 cell lines.

Benzophenone activated yeast strains carrying plasmids pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Ci*LBD.VP16 (Table 5.1). However, negative control strains also showed induced β-galactosidase activity (Figure 5.3D). These results suggest that β-galactosidase activity is induced by a process that is not dependant on binding of benzophenone to the tunicate VDR/PXRα LBD. In addition, two positive strains (pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Bs*LBD.VP16) showed concentration-dependent reduction of β-galactosidase activity (Figure 5.3D). Examination of OD_{620} values indicated a toxic effect towards yeast cells only in one strain (pGAL4.*Bs*LBD.VP16), while all other strains showed normal growth rates at high concentrations of benzophenone (Appendix 17C). The EC_{50} values obtained from two of the strains (pGAL4.*Ci*LBD∆31.VP16 and pGAL4.*Ci*LBD.VP16) need to be treated with caution as they may reflect a ligand-independent activation (Table 5.1). Identification of benzophenone as a *C. intestinalis* VDR/PXRα ligand is consistent with previous research which showed that benzophenone interacts with vertebrate PXR LBDs (e.g. rat PXR) when using a yeast-two hybrid assay (Mikamo *et al.* 2003).

Collectively, these results suggest the potential of EDCs to interact with tunicate PXR orthologues. Endocrine disrupting chemicals such as benzophenone, triclosan and 4-methylbenzylidene camphor are important environmental pollutants which are frequently detected in rivers, lakes and coastal areas (Kolpin *et al.* 2002; Lindstrom *et al.* 2002; Langford & Thomas 2008). These environmental contaminants can exhibit their endocrine disruptor activities by altering PXR-regulated steroid hormone metabolism. Therefore, yeast

bioassays utilising PXR LBDs may represent a valuable tool to detect EDCs with PXR-like activities in the environment (Mikamo *et al.* 2003). Further research is required to validate the data presented here due to the high variability and limited assay sensitivity to some EDC compounds.

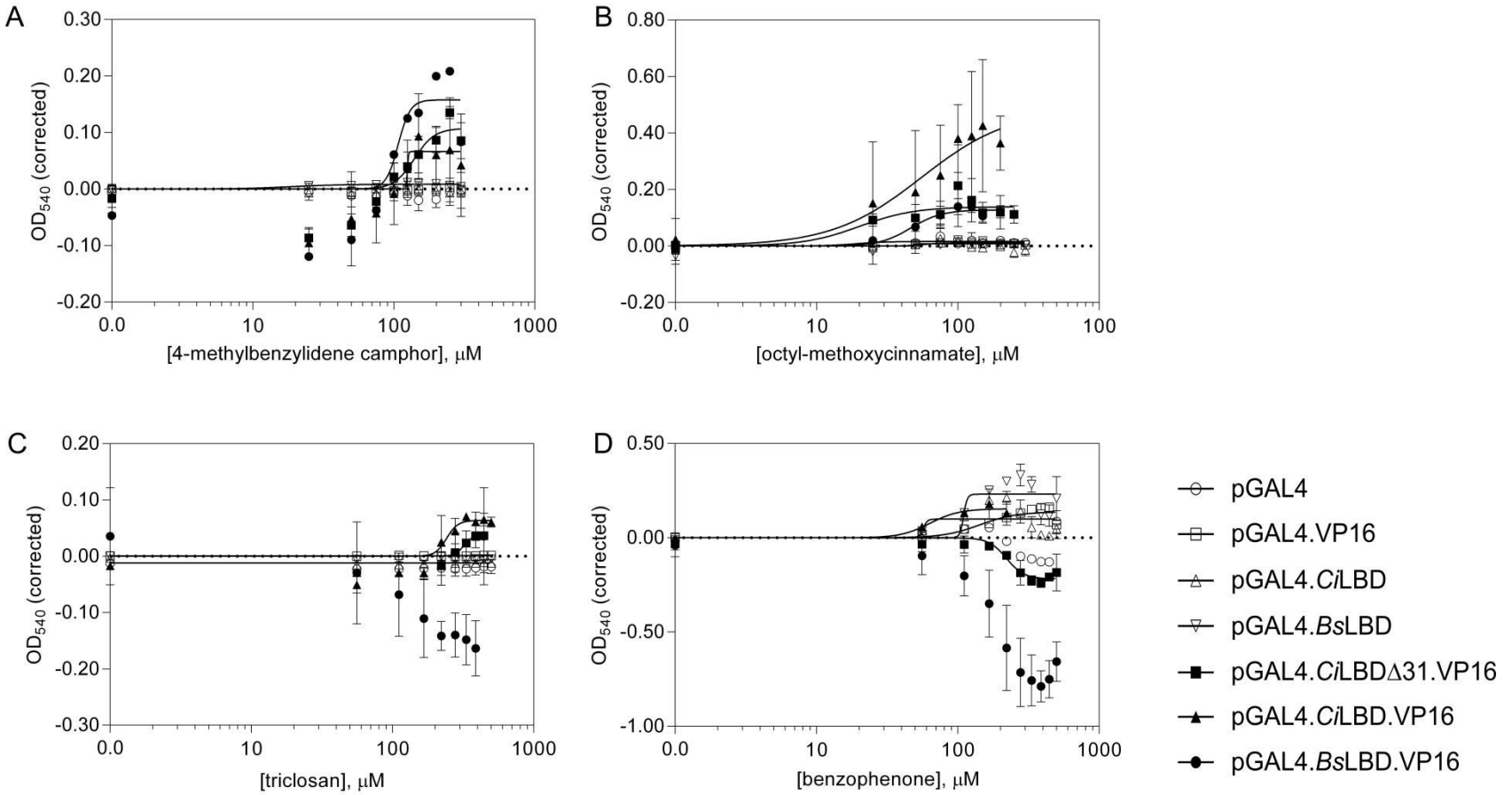

Figure 5.3 Activities of endocrine disrupting chemicals tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) 4-methylbenzylidene camphor, (**B**) octyl-methoxycinnamate, (**C**) triclosan and (**D**) benzophenone. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 24 h or 48 h (Table 5.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

5.3.1.3 Activity of fungicides

Antifungals are commonly used as pesticides and pharmaceuticals and can pose a significant risk to terrestrial and aquatic organisms (Kookana *et al.* 1998; Komarek *et al.* 2010; Peng *et al.* 2012). Among the three antifungal chemicals tested in this study (Figure 5.4), ketoconazole, an antifungal agent used in human and veterinary pharmaceuticals (Zarn *et al.* 2003), was the most potent activator of both tunicate VDR/PXRα yeast bioassays generating EC_{50} values in the mid nM range (Table 5.1). Yeast strains expressing the *C. intestinalis* VDR/PXRα LBD were consistently activated by ketoconazole (EC_{50} = 337–400 µM, CV = 5–18%; Table 5.1). These results are comparable to *in vivo* studies showing acute toxicity of ketoconazole towards *D. magna* neonates (EC_{50} = 2.8 µM; Haeba *et al*. 2008). In contrast, the human PXR LBD was not activated by ketoconazole when tested in a transactivation assay using HepG2 cell lines (Sinz *et al.* 2006). This may be attributed to the fact that ketoconazole acts as a general inhibitor of human PXR by disrupting the interaction of human PXR with the co-activator, steroid receptor co-activator-1 (SRC-1; Huang *et al*. 2007). A SRC-1 orthologue could not be identified in the *C. intestinalis* genome sequence (A. Fidler, *unpublished*) which may allow activation of the tunicate VDR/PXRα LBD yeast bioassays by ketoconazole. These data suggest that the yeast bioassay may be used to detect pharmaceutical drugs that cannot be detected in mammalian cell lines expressing human PXR (Sinz *et al.* 2006). Detection of ketoconazole and other pharmaceutical compounds in the aquatic environment is of increasing importance because they can be relatively persistent leading to bioaccumulation in surface and waste waters (Kahle *et al.* 2008; Lindberg *et al.* 2010; Peng *et al.* 2012). Long-term consumption of these environmental pollutants can cause adverse health effects in most organisms, including humans (Oros *et al.* 2003). For example, ketoconazole has been implicated in affecting the endocrine systems of aquatic vertebrates since they interact with several cytochrome P-450 enzymes such as CYP19 which takes part in hormone communication pathways (Zarn *et al.* 2003; Hasselberg *et al.* 2008).

The agricultural fungicide 2-phenylphenol was also active in yeast bioassays carrying the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD (pGAL4.*Ci*LBD.VP16, EC_{50} = 79 µM; pGAL4.*Bs*LBD.VP16, EC_{50} = 26 µM; Table 5.1). The responses were sigmoidal (Figure 5.4B) and relatively consistent (CV = 18–37%; Table 5.1). Similarly, human PXR has been shown to be activated by 2-phenylphenol in a transactivation assay using COS-7 simian kidney cells which was slightly more sensitive than the yeast bioassay (EC_{20} = 9.8 µM; Kojima *et al*. 2011).

The fungal antibiotic radicicol, a potent and selective inhibitor of heat shock protein 90 (HSP90; Feldman *et al*. 2009), reliably induced β-galactosidase enzymatic activity in strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 (CV = 9–17%; Table 5.1, Figure 5.4C). Identification of radicicol as an activator of both tunicate VDR/PXRα LBDs tested (EC_{50} = 9.5–13 µM; Table 5.1) was consistent with its reported toxicity using tunicate larval metamorphosis bioassays (*Ciona savignyi* IC_{99} = 2.2 µM, *Boltenia villosa* IC_{99} = 2.5 µM) suggesting that it may be toxic towards a wide range of chordates, including tunicates (Bishop *et al.* 2002; Cahill *et al.* 2012). These data indicate that the yeast bioassay may be a useful tool for monitoring these potent toxicants in the marine environment.

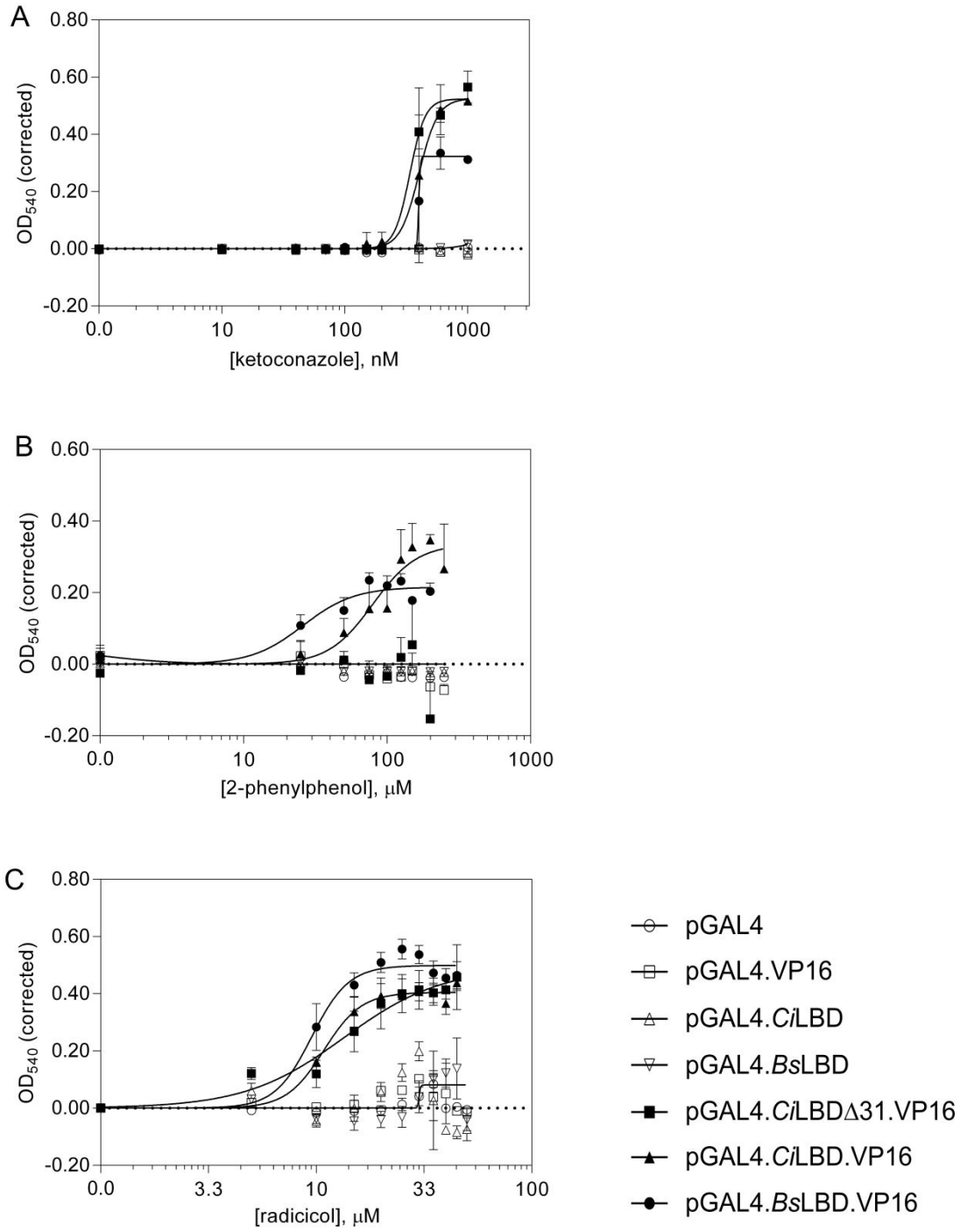

Figure 5.4 Activities of fungicides tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) ketoconazole, (**B**) 2-phenylphenol and (**C**) radicicol. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 24 h or 48 h (Table 5.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

5.3.1.4 Activity of insecticide

One of the most commonly used active ingredients in insect repellents, *n*,*n*-diethyl-*m*-toluamide (DEET), was tested for its ability to activate the tunicate yeast bioassays. Yeast strains expressing the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16 and pGAL4.*Ci*LBD.VP16) showed concentration-dependent induction of β-galactosidase activity (Table 5.1, Figure 5.5A). In contrast, a negative effect on β-galactosidase induction was observed in strains expressing the *B. schlosseri* VDR/PXRα LBD (pGAL4.*Bs*LBD.VP16; Figure 5.5A). This suppression effect was also apparent in reduced OD_{620} values at high concentrations of DEET, suggesting a general toxicity towards this particular yeast strain (Appendix 17D). The EC_{50} values observed in this *in vitro* yeast bioassay (54–99 µM; Table 5.1) were comparable to previously reported toxicity measures towards *D. magna* (EC_{50} = 177–836 µM; Seo *et al*. 2005; Weeks *et al*. 2012), the green alga *Pseudokirchnierella subcapitata* (EC_{50} = 21 µM; Harada *et al*. 2008) and the marine luminescent bacterium *Photobacterium phosphoreum* (EC_{50} = 355 µM; Kaiser & Palabrica 1991) using *in vivo* acute toxicity bioassays. Although DEET is present in the aquatic environment world-wide (e.g. drinking water, streams, open seawater, groundwater and treated effluent), concentrations in surface waters are several hundreds of times lower than those toxic to aquatic organisms, and the probability for adverse effects to aquatic species is low (Costanzo *et al.* 2007; Weeks *et al.* 2012). However, identification of DEET as an activator of the *C. intestinalis* VDR/PXR LBD suggests that it may interact with invertebrate PXR orthologues. Due to the close phylogenetic relationship of tunicates and vertebrates (Delsuc *et al.* 2008), it could have adverse effects on vertebrate physiology via interaction with PXR.

5.3.1.5 Activity of pharmaceutical drugs

Interest in the presence of pharmaceutical drugs in the environment has increased significantly over the last decade because hundreds of tonnes of these compounds are dispensed every year. For example, diclofenac sodium salt (diclofenac) is one of the most abundant pharmaceutically active compounds present in the water cycle (Heberer *et al.* 2002). Diclofenac induced β-galactosidase enzymatic activity in strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 (Table 5.1, Figure 5.5B) albeit with high variability (CV = 38–44%; Table 5.1). The yeast strain expressing the *B. schlosseri* VDR/PXRα LBD, pGAL4.*Bs*LBD.VP16, was more strongly activated by diclofenac than the strain expressing the *C. intestinalis* VDR/PXRα LBD, as indicated by the plateau levels of β-galactosidase production and EC_{50} values (Figure 5.5A, Table 5.1). The EC_{50} values observed in this study (11–91 µM; Table 5.1) were in the same range as those previously reported for zebrafish (*Danio rerio*) using embryo toxicity tests (EC_{50} = 17 µM), *D. magna* using acute immobilisation tests (EC_{50} = 213 µM) and planktonic green alga (*Desmodesmus subspicatus*) using growth inhibition tests (EC_{50} = 226 µM; van den Brandhof & Montforts 2010). Since these concentrations are classified as being potentially harmful to aquatic organisms (Cleuvers 2004), the yeast bioassay may provide an alternative method of detecting pharmaceuticals and harmful synthetic chemicals in the environment.

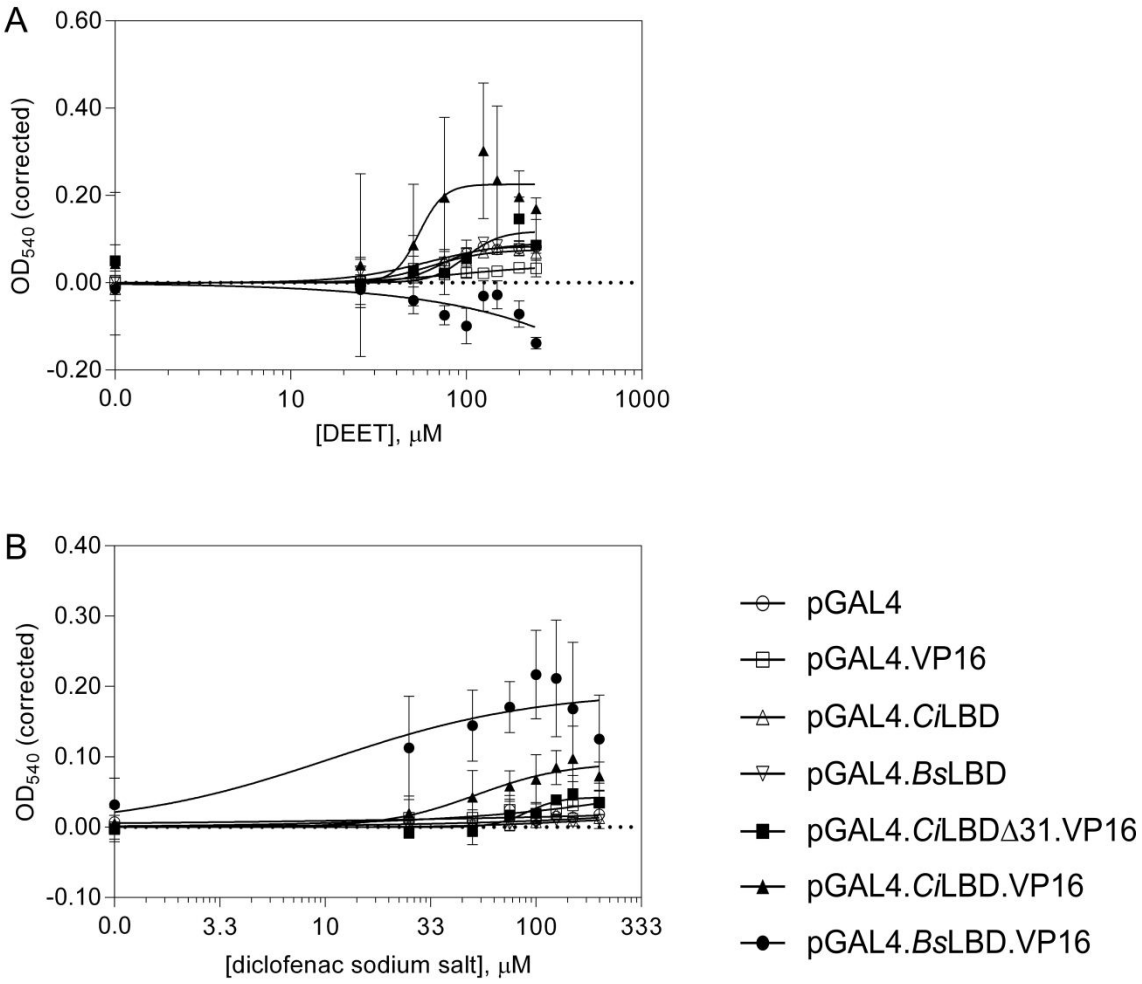

Figure 5.5 Activities of *n*,*n*-diethyl-*m*-toluamide (DEET) and diclofenac sodium salt tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of (**A**) *n*,*n*-diethyl-*m*-toluamide (DEET) and (**B**) diclofenac sodium salt. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h (Table 5.1). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

5.3.2 Activity of commercial oil dispersants in the tunicate yeast bioassays

Based on the findings that synthetic chemicals (e.g. pharmaceuticals, pesticides, etc.) can be detected using the yeast bioassay, five complex chemical mixtures that are frequently used to mitigate marine oil spills were analysed (Table 5.2). Three of the tested dispersants showed complete sigmoidal concentration-response curves in the tunicate yeast bioassays (Table 5.2, Figure 5.6A–C). Dispersant D only activated the *C. intestinalis* VDR/PXRα LBD-based yeast bioassay but not the *B. schlosseri* VDR/PXRα equivalent (Figure 5.6D). Dispersant E showed no ligand-dependent induction of β-galactosidase activity in any of the yeast strains tested (Figure 5.6E). The CVs of the EC_{50} values for oil dispersants that induced β-galactosidase activity in yeast strains were within the acceptable range of 6–19% (Table 5.2). These data (EC_{50} = 67–207 mg/L) were comparable to previous *in vivo* studies using the marine rotifer *Brachionus plicatilis* (LC_{50} = 0.5–40 mg/L; Kujawinski *et al.* 2011) indicating that oil dispersants are highly toxic towards micro-zooplankton. In addition, it has been shown that oil dispersants are also toxic towards a wide range of marine organisms including anemones, corals, crustaceans, starfish, molluscs, fish, and sperm whale (Wise & Wise 2011; Goodbody-Gringley *et al.* 2013; Almeda *et al.* 2014). In addition, three commercially available dispersants have been shown to increase cytotoxicity *in vitro* (LC_{50} = 250–400 mg/L) using HepG2 cells in a hepatotoxicity screening assay suggesting that dispersants can also cause adverse health effects in humans (Bandele *et al.* 2012; D'Andrea & Reddy 2013). Further research is needed before clear conclusions can be drawn regarding the toxicity of dispersants towards marine organisms. However, efficient monitoring tools that allow early detection of these environmental contaminants are required to assist in mitigating the environmental impact and long-term consequences of these surfactants.

Table 5.2 Activation of the tunicate yeast bioassays by five commercial oil dispersants.
Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in mg/L. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All oil dispersants were dissolved in ethanol.

Oil dispersant	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
			EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Dispersant A [a]	Surfactant	Dr Tremblay	101 (88–117)	11	67 (53–83)	10	118 (21–660)	6
Dispersant B [a]	Surfactant	Dr Tremblay	163 (138–193)	13	151 (120–191)	16	169 (107–266l)	6
Dispersant C [a]	Surfactant	Dr Tremblay	137 (105–179)	11	126 (104–152)	19	207 (161–266)	12
Dispersant D [a]	Surfactant	Dr Tremblay	92 (63–135)	14	146 (123–174)	10	NI (NI)	DNC
Dispersant E [a]	Surfactant	Dr Tremblay	NI (DNC)	DNC	NI (DNC)	DNC	~11 (DNC)	30

Abbreviations: DNC, did not compute; NI, no induction of β-galactosidase enzymatic activity.

[a] Strains were incubated for 48 hours.

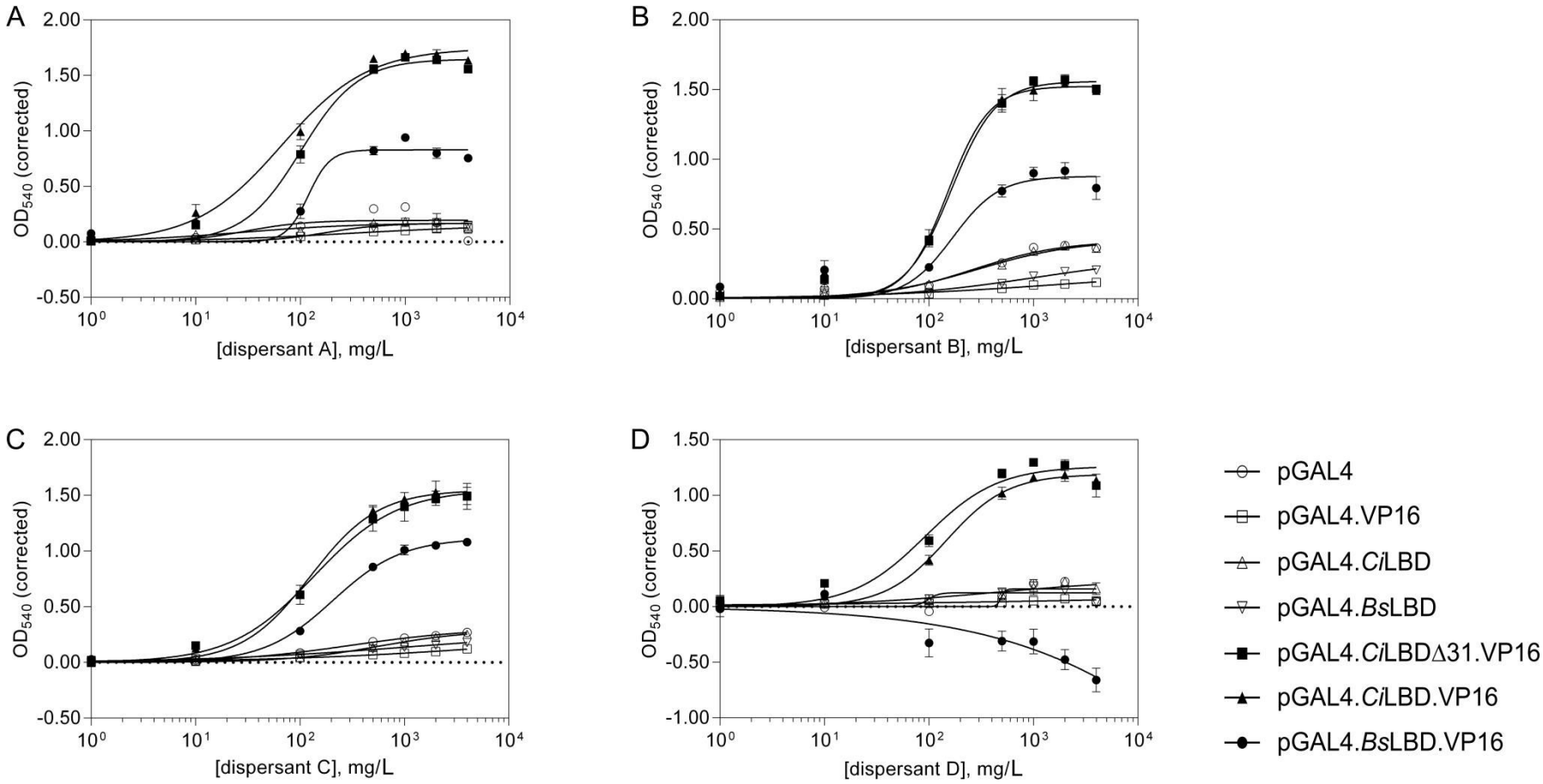

Figure 5.6 Activities of oil dispersants tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of: (**A**) dispersant A, (**B**) dispersant B, (**C**) dispersant C and (**D**) dispersant D. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h (Table 5.2). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

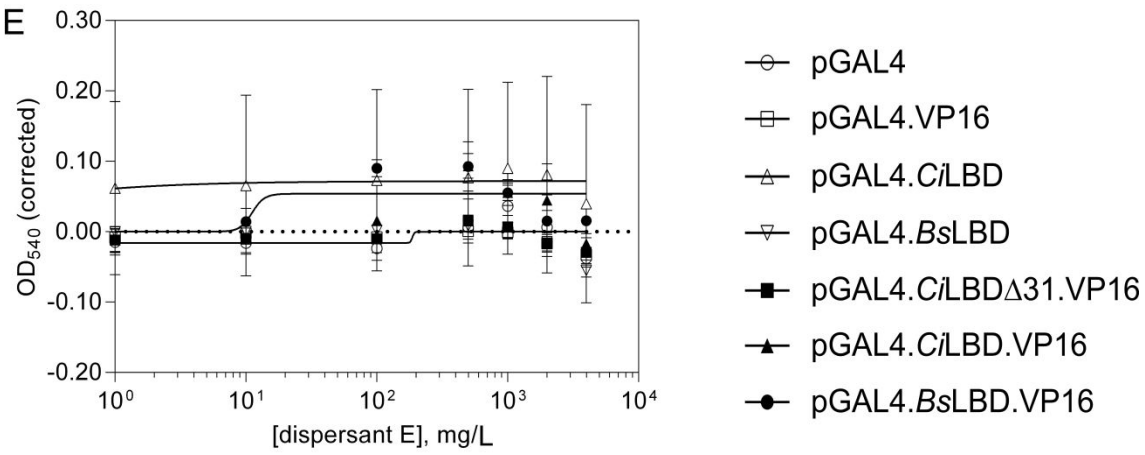

Figure 5.6 (cont.) Activities of oil dispersants tested in the yeast bioassays.

Responses of yeast strains carrying one of seven different pGBKT7-based expression vectors during exposure to a range of concentrations of (**E**) dispersant E. β-galactosidase enzymatic activities, as measured by $OD_{540\ (corrected)}$ values, were determined after 48 h (Table 5.2). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

5.3.3 Characteristics of the yeast bioassays

Although the yeast bioassays were activated by a wide range of synthetic chemicals, there was considerable variation of activity between different test compounds (Table 5.1). This may be due to structural differences between the chemicals analysed, which are likely to affect the receptor-binding affinity. As has been previously reported for a *C. intestinalis* VDR/PXRα LBD-based mammalian cell line assay (Fidler *et al.* 2012; Chapter Three), the tunicate VDR/PXRα LBD-based yeast bioassay appeared to be more sensitive towards structurally complex chemicals (e.g. ketoconazole, radicicol; Figure 5.1I&K and 5.4A&C) Among the remaining compounds, a clear correlation between assay sensitivity and compound structure could not be identified. For example, yeast bioassays were highly sensitive towards butylated hydroxytoluene but were less sensitive towards the structurally similar 2-phenoxyethanol (Figure 5.1A&C and 5.2A&C, Table 5.1). Another possible explanation for the high assay variability is the solubility of the test compounds in ethanol. 2-phenylphenol, 4-chloro-3,5-

dimethylphenol and triclosan are practically insoluble in ethanol. Consequently, follow-up studies were undertaken where all 13 synthetic chemicals were dissolved in DMSO and retested in the yeast bioassays. Compounds that were dissolved in DMSO generally showed reduced sensitivity (higher EC_{50} values) but with increased variability (greater CV) when compared to data generated from compounds dissolved in ethanol (Appendix 16). It also appeared that the high assay variability was more evident with the low level β-galactosidase enzymatic activities (corrected OD_{540} <0.2) as indicated by shallow log-dose response curves (Table 5.1, Figures 5.2–5.5).

To overcome these differences in sensitivity, additional organic solvents (e.g. acetone) should be considered in future bioassays as it is likely to result in improved assay sensitivity and consistency for certain compounds. In addition, an increase in replicate numbers (e.g. five replicates) and robotic liquid handling may improve reliability and repeatability of the bioassay as high intra-assay variability often reflects issues arising from pipetting very small volumes (Schultheiss & Stanton 2009).

The data from this chapter shows that tunicate yeast bioassays represent an accurate, sensitive and reliable alternative to traditional *in vivo* toxicity screening assays for some of the compounds tested (e.g. butylated hydroxytoluene, ketoconazole and radicicol). Based on biologically relevant receptors, the tunicate VDR/PXRα LBD-based yeast bioassay may provide consistent proxies for assessing the effects of environmental contaminants on invertebrate members of coastal ecosystems (Stewart *et al.* 2014).

One of the main advantages of the yeast bioassay over *in vivo* assays is the ability to assess the toxicity of complex mixtures rather than single, well-characterised pure compounds. Analysis of complex mixtures is particularly important when assessing toxicity of environmental pollutants because the additive interaction of these compounds in mixtures can cause different biological effects compared to pure compounds (Kinani *et al.* 2010; Bandele *et al.* 2012). Additive mixture effects have been demonstrated *in vitro* for anti-androgenic pesticides, for compounds with estrogenic properties, and for compounds tested *in vivo* (Payne *et al.* 2000; Birkhoj *et al.* 2004). Low exposure to multiple EDCs with similar modes of action has also been shown to deliver a

toxicological response, even though the effect of the individual compounds was below the limit of detection (Silva *et al.* 2002; Christiansen *et al.* 2008). The tunicate yeast bioassay may provide an alternative method to fill this gap.

5.4 Conclusion

The tunicate VDR/PXRα LBD-based yeast bioassays tested in this study were activated by a wide range of structurally diverse synthetic chemicals. These chemicals included bioactive compounds frequently used as preservatives in; foods, drugs and cosmetics, as UV-filters in personal care products, and as antifungal agents in pesticides and pharmaceuticals. This is of particular importance because many of these chemicals are frequently discharged into the environment, and can disrupt normal physiological functions (Oros *et al.* 2003; Kinani *et al.* 2010; Stewart *et al.* 2014).

This chapter highlights the feasibility of using tunicate VDR/PXRα LBD-based yeast bioassays for detecting anthropogenic chemicals. Future research should focus on testing mixtures, formulations and environmental samples in the yeast bioassays. Combining these *in vitro* yeast bioassays with detailed chemical analyses will provide a powerful tool to detect and identify bioactive compounds within complex samples (Eggen & Segner 2003). An important aspect to be considered when developing the tunicate yeast bioassay into an environmental monitoring tool is the extreme permissiveness of the tunicate VDR/PXRα LBD towards a wide range of ligands (Watkins *et al.* 2001; Watkins *et al.* 2003; Chrencik *et al.* 2005). It is likely that the yeast bioassay is less sensitive towards certain classes of pollutants (e.g. EDCs) when compared to other NR-reporter assays (e.g. ERα reporter assay), which use highly selective NR LBDs that bind a structurally limited range of ligands (Routledge & Sumpter 1996; Zhang *et al.* 2004; Krasowski *et al.* 2005). However, the ability to detect a wide range of structurally diverse bioactive chemicals makes the tunicate yeast bioassay a valuable tool for high-throughput screening of environmental samples.

5.5 References

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Coastal monitoring buoy used to remotely collect environmental data on the water quality of Tasman Bay, New Zealand.
Photo provided by Cawthron Institute.

CHAPTER SIX

Conclusions

6.1 Thesis synthesis

Detailed discussions are provided at the end of each chapter of this thesis. The purpose of this section is to collate and summarise the major conclusions arising from the key findings and recommend areas for further research.

The research outcomes from this thesis demonstrate that a specific group of ligand-activated transcription factors, namely tunicate xenobiotic receptors, may provide a source of sensor elements that have been pre-moulded by natural selection for detecting bioactive chemicals. High intra-taxa genetic diversity within tunicate xenobiotic receptor genes, orthologues to vertebrate vitamin D receptor (VDR) and pregnane X receptor (PXR), was reported in Chapter Two. These data supported the notion that tunicate VDR/PXR receptors might enhance binding of dietary exogenous bioactives/toxins typically encountered by these organisms. This provided the foundation for the development of a generic bioassay for assessing the activity of these biological compounds (Chapter Three). Recombinant yeast strains were developed in which ligand-dependent activation of tunicate VDR/PXRs was transduced into easily quantifiable phenotypic changes in the yeast strains. In this thesis, I showed that tunicate yeast bioassays have the potential for detecting microalgal biotoxins (Chapter Three), bioactive compounds that may provide templates for drug development (Chapter Four) and environmental contaminants (Chapter Five).

Previously, sequence comparisons between vertebrate taxa have suggested positive selection is acting on ligand-binding domain (LBD) coding sequences of PXR and its orthologues (Zhang *et al.* 2004; Krasowski *et al.* 2005). In Chapter Two, high intra-species sequence variation in VDR/PXR LBD coding sequences of two tunicates, *Ciona intestinalis* and *Botryllus schlosseri*, was reported. To

characterise the effect of natural selection at the molecular level, non-synonymous variation (i.e. amino acid changing) was compared to synonymous changes (McDonald & Kreitman 1991) which indicated a strong purifying selection process. While this result was unexpected, recent studies have suggested that multiple evolutionary forces can affect amino acid substitution rates (Bromham 2009; Lourenco *et al.* 2013), with one critical factor being mutation. An elevated per-year mutation rate, due to a large effective population size and a short generation time, may explain the extremely highly levels of genetic diversity within tunicates (Kimura 1983; Lynch 2008; Tsagkogeorga *et al.* 2012; Berná & Alvarez-Valin 2014). In addition, the rate of environmental change and dimensionality of the phenotypic space (organism complexity) can affect adaptive rates in some species (Lourenco *et al.* 2013). While it is difficult to evaluate how these factors translate to changes in fitness, it is assumed that fast evolving tunicates are able to adapt rapidly to environmental challenges (Tsagkogeorga *et al.* 2012). Adaptive genetic signatures (e.g. mutation rates over time) of tunicates can be very informative of changes to our marine environment because the influence of environmental factors can lead to accumulation of genetic variation (Dalziel *et al.* 2009; Barrett & Hoekstra 2011). One such factor is climate change which imposes novel selection pressures on organisms by altering the abiotic and biotic environmental conditions. Although studies demonstrating genetic adaptation to climate change-mediated selection are still scarce (Merila 2012; Merila & Hendry 2014), the frequencies of some well-characterised genetic polymorphisms in *Drosophila* species have been shown to shift with climate change (Umina *et al.* 2005; Balanya *et al.* 2006).

Alternatively, a selective advantage in the marine environment may be facilitated through different processes. *Ciona intestinalis* VDR/PXR protein variants consisting of a DNA-binding domain (DBD) but lacking a LBD were identified in this study. The persistence of these variants may indicate constitutive expression of detoxification genes, thus providing a biochemical protection mechanism in a ligand-independent manner. In addition, tunicate genomes encode multiple VDR/PXR paralogues, with each subtype potentially binding a differing range of ligand structures (Dehal *et al.* 2002; Denoeud *et al.* 2010; Voskoboynik *et al.* 2013).

In previous studies, Fidler *et al*. (2012) demonstrated that LBDs from a tunicate xenobiotic receptor can be activated by microalgal biotoxins when expressed in mammalian cell lines. In Chapter Three, *C. intestinalis* and *B. schlosseri* VDR/PXRα LBDs were functionally expressed in recombinant yeast (*Saccharomyces cerevisiae*) strains as chimeric proteins. Three biotoxins, produced by microalgae that are expected to be part of the tunicate diet, were identified as putative VDR/PXRα LBD ligands. This supported the idea that the natural tunicate VDR/PXRα receptor may be activated by exogenous compounds relevant to its marine environment. The sensitivity of the yeast bioassay towards both microalgal biotoxins and synthetic compounds appeared to depend on several factors, such as the organic solvent used, duration of exposure and type of recombinant protein expressed (e.g. *C. intestinalis* verses *B. schlosseri* VDR/PXRα). This implies that assay sensitivity can be improved by changing or varying these factors (Eichbaum *et al.* 2014). Assay sensitivity may also be improved by using a different type of reporter gene. For example, yeast-enhanced green fluorescence protein and luciferase reporter assays are more sensitive than *lacZ* (Fan & Wood 2007) and these could be investigated in the future for this bioassay.

The development of robust high-throughput screening (HTS) assays for identifying natural marine bioactive compounds for pharmaceutical purposes is technically challenging (Martins *et al.* 2014). Chapter Four, showed that the yeast bioassays were highly sensitive towards a small number of marine and terrestrial bioactive compounds. These yeast bioassays have a number of characteristics that may be beneficial for pharmaceutical screening. The orthology of the tunicate VDR/PXRα with vertebrate PXR (Ekins *et al.* 2008) suggests that compounds active in the yeast bioassay may also affect vertebrate physiology. This is an important consideration when developing bioassays for drug discovery, because the natural biological activities of putative drug compounds influence their potential medical applications (Imhoff *et al.* 2011; Martins *et al.* 2014). The yeast bioassays are also well-suited for HTS, which is the most widely used screening approach for the identification of novel drug lead compounds (Hughes *et al.* 2011; Martins *et al.* 2014). Therefore, if sensitivity and reliability of yeast bioassays can be improved in future, these assays may provide a template for the development of cost-

effective methods for the detection of bioactive compounds during the early phases of drug discovery.

Recently, a new class of environmental pollutants, termed emerging contaminants (ECs), has received scientific and public interest because they may pose a health risk to many organisms, including humans (Gavrilescu *et al.* 2015). In Chapter Five, a wide range of synthetic chemicals, such as pesticides, pharmaceuticals and surfactants, were shown to activate the tunicate yeast bioassays. Although the responses for some of the aforementioned compounds were highly variable, the *in vitro* bioassay has the potential to be a reliable and inexpensive tool for monitoring chemical mixtures such as surfactants (e.g. oil dispersants; Bandele *et al*. 2012). By evaluating data generated in the yeast *in vitro* bioassay, it may be possible to predict the effects of environmental surfactants on coastal invertebrates, especially when combined with *in vivo* toxicity studies. This is of particular importance since most *in vitro* bioassays focus on assessing the physiological effects that environmental contaminants can cause in humans, while overlooking the far-reaching impacts on aquatic organisms.

6.2 Future research

The data presented in this thesis provide a foundation for further investigations into the molecular evolution, population genetics and function of tunicate xenobiotic receptors thought to be involved in the detection of marine bioactive compounds. If dietary bioactive xenobiotics act as selective agents in shaping the structure of tunicate VDR/PXR LBDs, then it is reasonable to expect that exposure to different dietary toxins in species occupying different ecological niches will be reflected in intra-species sequence variation in VDR/PXR orthologues. This is supported by the fact that geographical location and exposure to toxic compounds can generate different mutational variants of a species (Whitehead *et al.* 2003). Genetic analysis of tunicate VDR/PXR orthologues from populations from different geographical locations and ecological niches may allow identification of targets of adaptive natural selection

(Li *et al.* 2008). Another possible approach could involve measuring differences in gene expression between taxa, by considering the hypothesis that at least some differences in the pattern or level of transcript abundance represent phenotypic traits contributing to adaptation (Ranz & Machado 2006).

The role of VDR/PXR orthologues in intact, living tunicates has not been determined and the toxicity of microalgal biotoxins towards tunicates is currently unknown. The development of a tunicate *in vivo* bioassay, using *C. intestinalis* adults, would assist in addressing these knowledge gaps. These assays would complement current *in vivo* bioassays used to investigate toxicological effects of chemicals on metamorphosis and larval development (Bellas *et al.* 2003; Cahill *et al.* 2012). Another interesting approach for studying the functional role of tunicate xenobiotic receptors could involve selective breeding of *C. intestinalis* adults that are homozygous for the VDR/PXR allelic variants that produce VDR/PXR proteins lacking a LBD. Exposure of these organisms to a range of known xenobiotics, including microalgal biotoxins, may reveal if VDR/PXR target genes are constitutively transcribed providing an advantage over ligand-activated transcription.

The tunicate yeast bioassay developed in this study represents a promising template for the development of a broad-spectrum screening tool which could be used for routine microalgal biotoxin detection and bioprospecting. To assess the bioassay's reliability, extracts of toxic shellfish should be tested for presence of microalgal biotoxins. It would be particularly useful to compare these *in vitro* data with detailed chemical liquid chromatography–mass spectrometry analyses to confirm the yeast bioassay's value in an applied context. If this can be achieved, then the yeast bioassay has the potential to significantly reduce the costs associated with current chemistry-based methods and may assist in the detection of unknown toxins (Humpage *et al.* 2010; Nicolas *et al.* 2014). This novel method may also be used by those with little expertise and who have only minimal capital to invest in equipment (i.e. developing countries).

To test the reliability of the yeast bioassay for bioprospecting, libraries of natural products and environmental extracts should be analysed for bioactive compounds and the resulting data compared with established ligand-binding assays used during drug discovery (Pinne & Raucy 2014). Compound libraries

are usually composed of crude extracts, simplified extract fractions and pure compounds for a well-balanced drug discovery programme (Kingston 2011). Crude extracts are complex mixtures of several compounds that may have synergistic interaction causing false negative read-outs (Parker *et al.* 2010). Due to the broad-spectrum activity of xenobiotic receptors, interference of other compounds that bind to the receptor is likely to cause false positives. Screening pre-fractionated libraries would be an effective strategy to avoid these problems (Carter 2011). To be applied in bioprospecting, the yeast bioassay needs to be amenable to HTS formats (Hughes *et al.* 2011). All yeast bioassay experiments conducted in this thesis were performed in 96-well plate formats which have the potential to be miniaturised to high-throughput 1536-well microplate formats (Rajasarkka & Virta 2011). The yeast bioassay could easily be adapted to robotic liquid handling, one of the main requirements for HTS. Assay miniaturisation and robotic liquid handling would likely reduce inter-plate variability, thus increasing the over-all reliability of the yeast bioassay. In addition, the *S. cerevisiae* host strain allows for refinements in the detection specificities and sensitivities of the yeast bioassay using laboratory-based *in vitro* mutagenesis and directed evolution (Chen & Zhao 2003). Using this approach VDR/PXR LBD variant sequences, generated by *in vitro* mutagenesis, can be selected for enhancement of growth rates in the presence of a cognate ligand. For example, Chen & Zhao (2003) used random *in vitro* mutagenesis combined with directed evolution to generate novel variants of the human estrogen receptor (ERα) LBD that had significantly modified ligand-binding properties.

To be used in environmental monitoring, it is crucial that the yeast bioassays meet analytical goals and/or regulatory guidelines. Most ECs generally occur in surface and ground waters at very low concentrations (e.g. ng/L to µg/L; Stewart *et al*. 2014). Thus, the relatively low sensitivity and reliability of the tunicate yeast bioassays towards a wide range of environmental contaminants is currently the major limitation. These limitations could be overcome by developing biosensors that are based on tunicate VDR/PXR receptors (Rodriguez-Mozaz *et al.* 2006; Holdgate *et al.* 2010; Senveli & Tigli 2013). The main advantage of biosensors is their cell-free nature, thereby removing the limitations associated with cell-based bioassays, such as the need for test

compounds to cross cell membranes (Norcliffe *et al.* 2013). Biosensors typically consist of a macromolecule immobilised on a surface via either covalent or strong non-covalent bonds (Fechner *et al.* 2010). An important consideration is that such attachments should not significantly influence the natural structure of the macromolecule or change its functionality in unpredictable ways (Fechner *et al.* 2010). A wide range of techniques exist to detect and quantify interactions between the immobilised macromolecules and potential ligands including; calorimetric, acoustic, electrical, magnetic and optical sensing techniques (Senveli & Tigli 2013). Development of such biosensor techniques utilising tunicate VDR/PXRα receptors as the sensor element may result in a more universal and generic detection system compared to yeast-based bioassays. Numerous nuclear receptor (NR) LBD-based biosensors have been developed for use in environmental monitoring (Dutta *et al.* 2007) and drug development (Fechner *et al.* 2010; Raucy & Lasker 2013). Among the established xenobiotic receptors, the human PXR LBD has been used successfully as the sensor element in a number of differing biosensor formats (Moore *et al.* 2000; Hill *et al.* 2011; Lin *et al.* 2014). These biosensors have confirmed a number of human PXR ligands such as; hyperforin, clotrimazole, ginkgolide A, SR12813 and 5b-pregnane-3,20-dione (Jones *et al.* 2000; Moore *et al.* 2000; Lin *et al.* 2014). The successful development of human PXR LBD-based biosensors supports the theoretical feasibility of using tunicate VDR/PXR LBDs in biosensor formats to screen for bioactive compounds. If routine production of correctly folded and soluble tunicate VDR/PXR LBDs can be achieved, they could be used in affinity chromatography to identify and isolate novel VDR/PXR ligands, as has recently been reported for the human PXR LBD (Dagnino *et al.* 2014).

Determining the three dimensional structure of the *C. intestinalis* and *B. schlosseri* VDR/PXRα receptor represents another important area of future research. Crystallisation of the VDR/PXR LBD in complex with a putative ligand may provide important insights into the permissiveness of these receptors as has been reported for vertebrate PXR (Wallace & Redinbo 2013; Wu *et al.* 2013). Previous studies suggested that the *C. intestinalis* VDR/PXR LBD may have narrower ligand selectivity when compared to vertebrate, particularly human, PXRs (Ekins *et al.* 2008; Fidler *et al.* 2012). While only structural data can address these questions, it would be interesting to study the function of the

second *C. intestinalis* VDR/PXR orthologue (VDR/PXRβ) using the recombinant yeast strains developed in this study. This gene had a higher genetic diversity than VDR/PXRα, and the predicted protein sequence suggested a larger LBD than its VDR/PXRα paralogue. Therefore, the VDR/PXRβ LBD may be able to bind larger compounds or a complete different set of ligands.

Due to the highly modular structure of the tunicate yeast bioassay, the VDR/PXR LBDs could be exchanged with virtually any suitable PXR orthologues from species other than *C. intestinalis* and *B. schlosseri*. By selecting, on the basis of taxonomy and ecology, the organism to source PXR LBDs from, it may be possible to tailor bioassays to search for bioactive compounds from differing sources. Ligand binding-domains of potential PXR orthologues have been identified in the genomes of other tunicates (e.g. six VDR/PXR-like genes were identified in *Oikopleura diocia;* Denoeud *et al.* 2010). There is no *a priori* reason why taxon selection should be restricted to the tunicates. For example, filter-feeding bivalves use a somewhat different mechanism for filtering seawater than tunicates. Therefore, these two groups ingest different profiles/size-ranges of marine microorganisms, thus being exposed to different toxins (Roje-Busatto & Ujević 2014). Some bioactive chemicals may be produced by marine organisms that adhere to hard surfaces. Benthic microalgae, such as the dinoflagellate *Gambierdiscus toxicus* can produce highly toxic compounds (e.g. ciguatera-associated toxins; Parsons *et al*. 2011). To detect such toxins, PXR LBDs from surface-grazing animals are likely to be more suitable for the yeast bioassay (Richter & Fidler 2014).

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Aquaculture buoys in Brightlands Bay, Tawhitinui Reach, Marlborough Sounds, New Zealand. Photograph provided by Cawthron Institute.

APPENDIX ONE

Appendix 1 Primers used for amplification of tunicate xenobiotic receptor orthologues.
(**A**) Primers used for the amplification of *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ), *C. intestinalis* actin (*Ci*actin) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα) partial coding sequences in polymerase chain reactions (PCRs). (**B**) Primers used for quantitative polymerase chain reactions (qPCR).

A

Gene	GenBank acc. no.		Primer sequence (5'–3')	Amplicon (bp)[a]
*Ci*VDR/PXRα	NM_001078379	Forward	AAGTATTTCTGATCCATCGCTGG	1326
		Reverse	GTGGGTTGAACTTCTTTAACAAGAGG	
*Ci*VDR/PXRβ	NM_001044366	Forward	ATGTCAAATCCTCAAGGCCCATC	1569[b]
		Reverse	CATGATGTTTTGAGCGAATAGC	
*Bs*VDR/PXRα	Not available	Forward	TGCGARGGCTGYAAAGGITTYTTCAG	1006
		Reverse	CACGTTGGGCATACATTCAAATAC	
*Ci*actin	AJ297725	Forward	CTTAGGCAGTTTTAATGCAAGCG	1454
		Reverse	TAGCAGCTGAAGCCGGTTTAGGAA	

Appendix 1 (cont.) Primers used for amplification of tunicate xenobiotic receptor orthologues.

B

Transcript	GenBank acc. no.		Primer sequence (5'–3')	Amplicon (bp)[a]	Reference
*Ci*VDR/PXRα	NM_001078379	Forward	GTTCACTTCACCGACATTATGGAG	103	This study
		Reverse	CGCGATTTGATCTTTTAGGTCC		
*Ci*VDR/PXRβ	NM_001044366	Forward	GCTAAACAACTTTTCCAACATTTC	100	This study
		Reverse	GGATAGTTGGACAAACTGTGGTA		
Ci actin	AJ297725	Forward	CTTCCTGACGGACAGGTTATCACC	213	Kulman, *et al*. 2006
		Reverse	TAGCAGCTGAAGCCGGTTTAGGAA		Kulman, *et al.* 2006

Abbreviations: *Ci*, *Ciona intestinalis*; *Bs*, *Botryllus schlosseri.*

[a] Predicted amplicon size in base pair (bp).

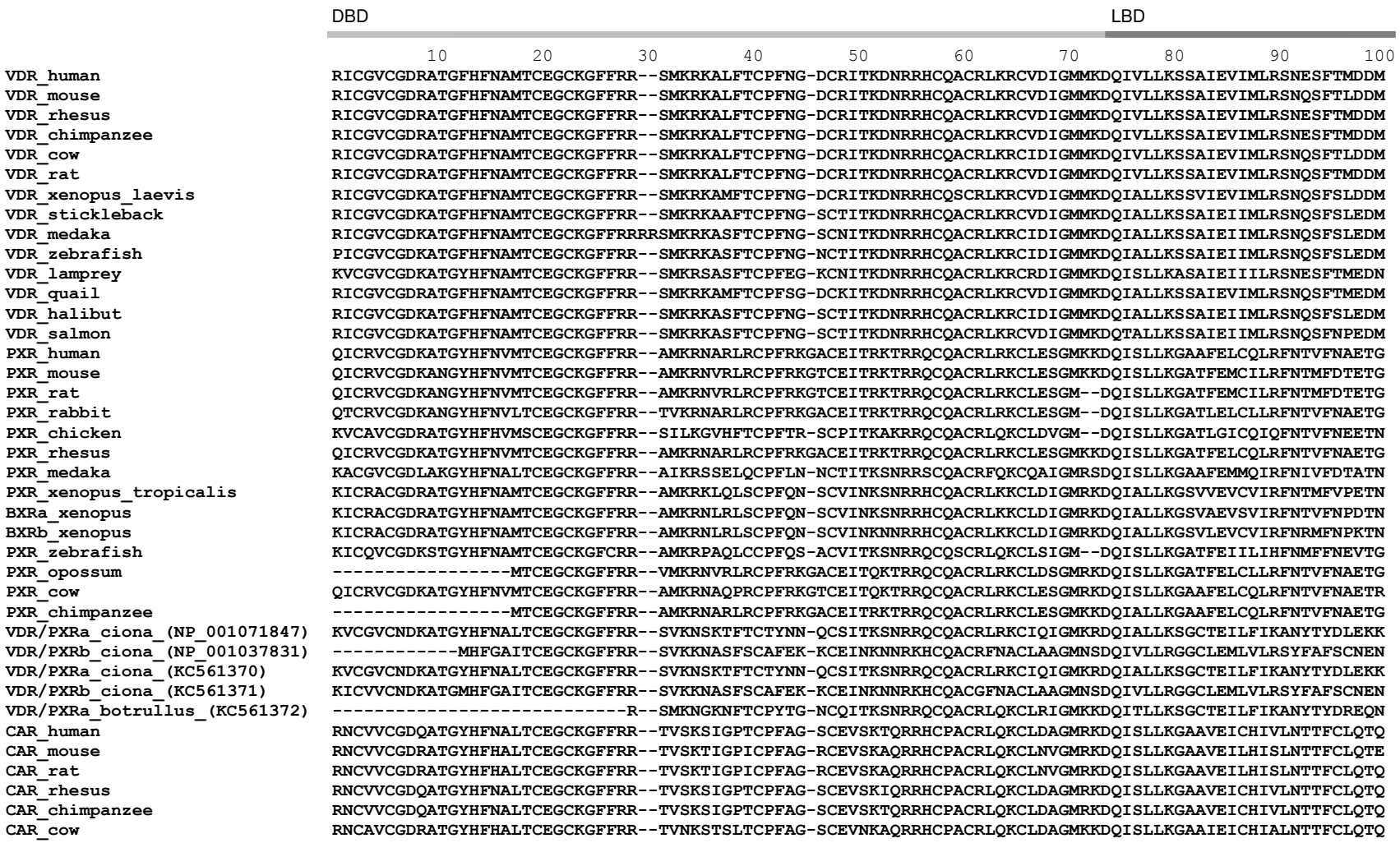
[b] PCR generated a longer *Ci*VDR/PXRβ amplicon than predicted by the primer positions in the reference sequence (NM_001044366).

APPENDIX TWO

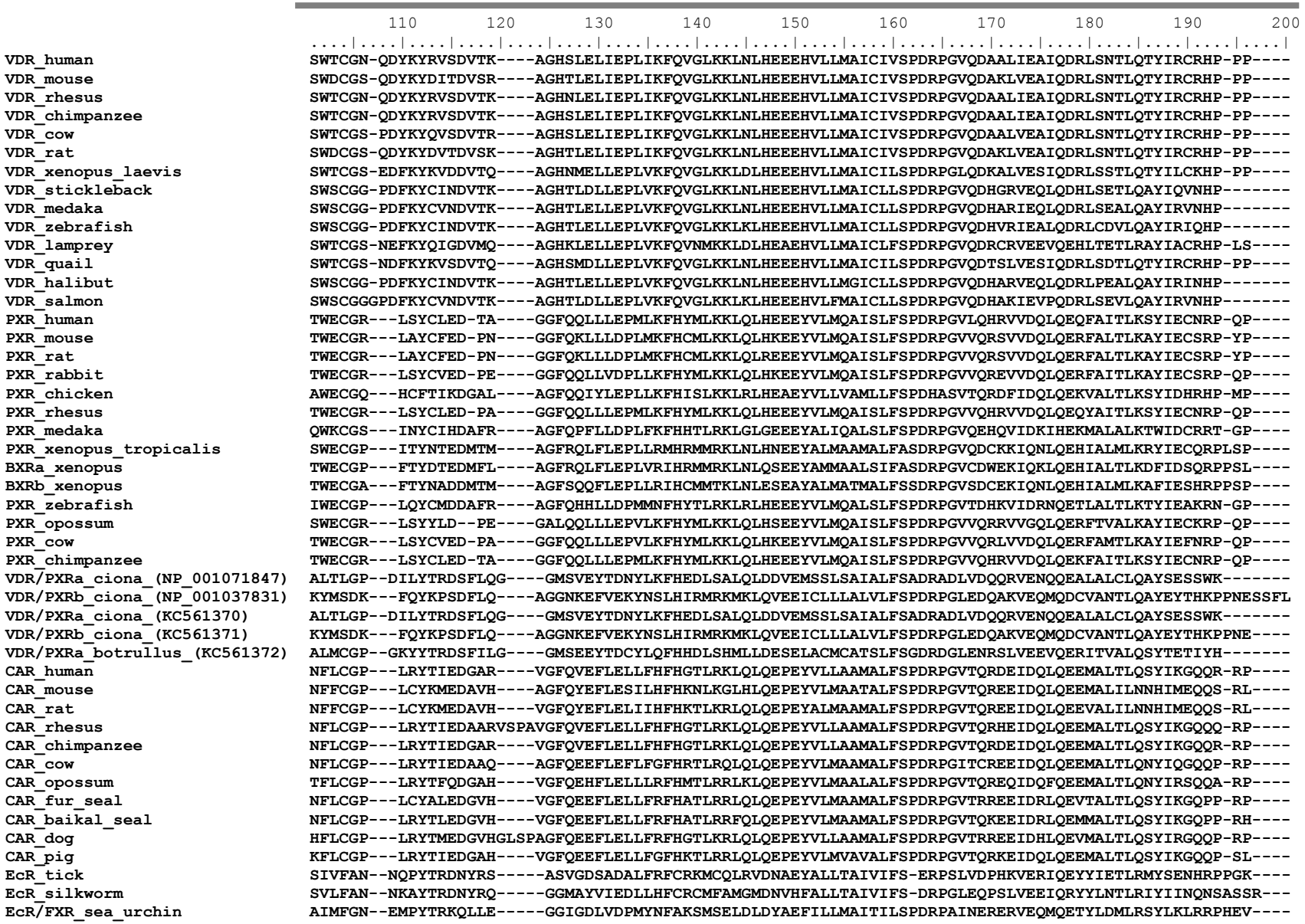
Appendix 2 Conserved domain predictions in three tunicate xenobiotic receptor protein sequences generated in this study. Predicted locations of DNA-binding domain (DBD) and ligand-binding domain (LBD) within the *Ciona intestinalis* vitamin D receptor/pregnane X receptor (*Ci*VDR/PXRα, KC561370), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, KC561371) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα, KC561372) conceptual proteins using the Simple Modular Architecture Research Tool (SMART; Letunic *et al*. 2012), the National Center for Biotechnology Information (NCBI) Conserved Domain (CD) database (NCBI CD; Marchler-Bauer *et al*. 2011) and the Protein Families database (Pfam; Punta *et al*. 2012).

	SMART		**NCBI CD**		**Pfam**	
	DBD	**LBD**	**DBD**	**LBD**	**DBD**	**LBD**
*Ci*VDR/PXRα	56–127	244–402	59–130	238–409	57–126	223–414
*Ci*VDR/PXRβ	1–59	320–480	2–88	312–480	1–58	305–480
*Bs*VDR/PXRα	2–42	153–311	1–44	145–311	1–40	135–325

APPENDIX THREE



```
CAR_opossum              RSCVVCGDRATGYHFHALTCEGCKGFFRR--TINKGMGLTCPFDQ-CCEVSKNQRRHCPACRLQKCLDVGMKKDQISLLKGAALEICHIELNTIFCPQSQ
CAR_fur_seal             RSCMVCGDRATGYHFHALTCEGCKGFFRR--TVSKNTGLTCPFAG-NCKVNKAQRRHCPACRLQKCLDAGMKKDQISLLKGAAVEICHIALNTTFCLQTR
CAR_baikal_seal          RSCMVCGDRATGYHFHALTCEGCKGFFRR--TVSKNTGLTCPFAG-SCKVNKAQRRHCPACRLQKCLDAGMKKDQISLLKGAAVEICHIALNTTFCLQTR
CAR_dog                  RNCMVCGDRATGYHFHALTCEGCKGFFRR--TVSKSTGLTCPFAG-SCKVNKAQRRHCPACRLQKCLDAGMRKDQISLLKGAAVEICHIALNTTFCLQTR
CAR_pig                  RNCAVCGDRATGYHFHALTCEGCKGFFRR--TVNKSTSLICPFAG-SCKVNKAQRRHCPACRLQKCLDAGMKKDQISLLKGAAVEICQIVLNTTFCLQTQ
EcR_tick                 ELCLVCGDRASGYHYNALTCEGCKGFFRR--SITKNAVYQCKYGN-NCDIDMYMRRKCQECRLKKCLSVGMRPDQITLLKACSSEVMMLRGARKYDVKTD
EcR_silkworm             ELCLVCGDRASGYHYNALTCEGCKGFFRR--SVTKNAVYICKFGH-ACEMDMYMRRKCQECRLKKCLAVGMRPDQITLLKASSSEVMMLRVARRYDAASD
EcR/FXR_sea_urchin       ELCLVCGDRASGFHYNALSCEGCKGFFRR--SITKNAKYNCTRGG-NCEMDMYMRRKCQECRLRKCREVGMLADQILLLKGSAIEVIMLRVALRYDRELD
```


110 120 130 140 150 160 170 180 190 200
VDR_human SWTCGN-QDYKYRVSDVTK----AGHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAALIEAIQDRLSNTLQTYIRCRHP-PP----
VDR_mouse SWDCGS-QDYKYDITDVSR----AGHTLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAKLVEAIQDRLSNTLQTYIRCRHP-PP----
VDR_rhesus SWTCGN-QDYKYRVSDVTK----AGHNLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAALIEAIQDRLSNTLQTYIRCRHP-PP----
VDR_chimpanzee SWTCGN-QDYKYRVSDVTK----AGHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAALIEAIQDRLSNTLQTYIRCRHP-PP----
VDR_cow SWTCGS-PDYKYQVSDVTR----AGHSLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAALVEAIQDRLSNTLQTYIRCRHP-PP----
VDR_rat SWDCGS-QDYKYDVTDVSK----AGHTLELIEPLIKFQVGLKKLNLHEEEHVLLMAICIVSPDRPGVQDAKLVEAIQDRLSNTLQTYIRCRHP-PP----
VDR_xenopus_laevis SWTCGS-EDFKYKVDDVTQ----AGHNMELLEPLVKFQVGLKKLDLHEEEHVLLMAICILSPDRPGLQDKALVESIQDRLSSTLQTYILCKHP-PP----
VDR_stickleback SWSCGG-PDFKYCINDVTK----AGHTLDLLEPLVKFQVGLKKLNLHEEEHVLLMAICLLSPDRPGVQDHGRVEQLQDHLSETLQAYIQVNHP-------
VDR_medaka SWSCGG-PDFKYCVNDVTK----AGHTLELLEPLVKFQVGLKKLNLHEEEHVLLMAICLLSPDRPGVQDHARIEQLQDRLSEALQAYIRVNHP-------
VDR_zebrafish SWSCGG-PDFKYCINDVTK----AGHTLELLEPLVKFQVGLKKLKLHEEEHVLLMAICLLSPDRPGVQDHVRIEALQDRLCDVLQAYIRIQHP-------
VDR_lamprey SWTCGS-NEFKYQIGDVMQ----AGHKLELLEPLVKFQVNMKKLDLHEAEHVLLMAICLFSPDRPGVQDRCRVEEVQEHLTETLRAYIACRHP-LS----
VDR_quail SWTCGS-NDFKYKVSDVTQ----AGHSMDLLEPLVKFQVGLKKLNLHEEEHVLLMAICILSPDRPGVQDTSLVESIQDRLSDTLQTYIRCRHP-PP----
VDR_halibut SWSCGG-PDFKYCINDVTK----AGHTLELLEPLVKFQVGLKKLNLHEEEHVLLMGICLLSPDRPGVQDHARVEQLQDRLPEALQAYIRINHP-------
VDR_salmon SWSCGGGPDFKYCVNDVTK----AGHTLDLLEPLVKFQVGLKKLKLHEEEHVLFMAICLLSPDRPGVQDHAKIEVPQDRLSEVLQAYIRVNHP-------
PXR_human TWECGR---LSYCLED-TA----GGFQQLLLEPMLKFHYMLKKLQLHEEEYVLMQAISLFSPDRPGVLQHRVVDQLQEQFAITLKSYIECNRP-QP----
PXR_mouse TWECGR---LAYCFED-PN----GGFQKLLLDPLMKFHCMLKKLQLHKEEYVLMQAISLFSPDRPGVVQRSVVDQLQERFALTLKAYIECSRP-YP----
PXR_rat TWECGR---LAYCFED-PN----GGFQKLLLDPLMKFHCMLKKLQLREEEYVLMQAISLFSPDRPGVVQRSVVDQLQERFALTLKAYIECSRP-YP----
PXR_rabbit TWECGR---LSYCVED-PE----GGFQQLLVDPLLKFHYMLKKLQLHKEEYVLMQAISLFSPDRPGVVQREVVDQLQERFAITLKAYIECSRP-QP----
PXR_chicken AWECGQ---HCFTIKDGAL----AGFQQIYLEPLLKFHISLKKLRLHEAEYVLLVAMLLFSPDHASVTQRDFIDQLQEKVALTLKSYIDHRHP-MP----
PXR_rhesus TWECGR---LSYCLED-PA----GGFQQLLLEPMLKFHYMLKKLQLHEEEYVLMQAISLFSPDRPGVVQHRVVDQLQEQYAITLKSYIECNRP-QP----
PXR_medaka QWKCGS---INYCIHDAFR----AGFQPFLLDPLFKFHHTLRKLGLGEEEYALIQALSLFSPDRPGVQEHQVIDKIHEKMALALKTWIDCRRT-GP----
PXR_xenopus_tropicalis SWECGP---ITYNTEDMTM----AGFRQLFLEPLLRMHRMMRKLNLHNEEYALMAAMALFASDRPGVQDCKKIQNLQEHIALMLKRYIECQRPLSP----
BXRa_xenopus TWECGP---FTYDTEDMFL----AGFRQLFLEPLVRIHRMMRKLNLQSEEYAMMAALSIFASDRPGVCDWEKIQKLQEHIALTLKDFIDSQRPPSL----
BXRb_xenopus TWECGA---FTYNADDMTM----AGFSQQFLEPLLRIHCMMTKLNLESEAYALMATMALFSSDRPGVSDCEKIQNLQEHIALMLKAFIESHRPPSP----
PXR_zebrafish IWECGP---LQYCMDDAFR----AGFQHHLLDPMMNFHYTLRKLRLHEEEYVLMQALSLFSPDRPGVTDHKVIDRNQETLALTLKTYIEAKRN-GP----
PXR_opossum SWECGR---LSYYLD--PE----GALQQLLLEPVLKFHYMLKKLQLHSEEYVLMQAISLFSPDRPGVVQRRVVGQLQERFTVALKAYIECKRP-QP----
PXR_cow TWECGR---LSYCVED-PA----GGFQQLLLEPVLKFHYMLKKLQLHKEEYVLMQAISLFSPDRPGVVQRLVVDQLQERFAMTLKAYIEFNRP-QP----
PXR_chimpanzee TWECGR---LSYCLED-TA----GGFQQLLLEPMLKFHYMLKKLQLHEEEYVLMQAISLFSPDRPGVVQHRVVDQLQEKFAITLKSYIECNRP-QP----
VDR/PXRa_ciona_(NP_001071847) ALTLGP--DILYTRDSFLQG----GMSVEYTDNYLKFHEDLSALQLDDVEMSSLSAIALFSADRADLVDQQRVENQQEALALCLQAYSESSWK-------
VDR/PXRb_ciona_(NP_001037831) KYMSDK---FQYKPSDFLQ----AGGNKEFVEKYNSLHIRMRKMKLQVEEICLLLALVLFSPDRPGLEDQAKVEQMQDCVANTLQAYEYTHKPPNESSFL
VDR/PXRa_ciona_(KC561370) ALTLGP--DILYTRDSFLQG----GMSVEYTDNYLKFHEDLSALQLDDVEMSSLSAIALFSADRADLVDQQRVENQQEALALCLQAYSESSWK-------
VDR/PXRb_ciona_(KC561371) KYMSDK---FQYKPSDFLQ----AGGNKEFVEKYNSLHIRMRKMKLQVEEICLLLALVLFSPDRPGLEDQAKVEQMQDCVANTLQAYEYTHKPPNE----
VDR/PXRa_botrullus_(KC561372) ALMCGP--GKYYTRDSFILG----GMSEEYTDCYLQFHHDLSHMLLDESELACMCATSLFSGDRDGLENRSLVEEVQERITVALQSYTETIYH-------
CAR_human NFLCGP---LRYTIEDGAR----VGFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSPDRPGVTQRDEIDQLQEEMALTLQSYIKGQQR-RP----
CAR_mouse NFFCGP---LCYKMEDAVH----AGFQYEFLESILHFHKNLKGLHLQEPEYVLMAATALFSPDRPGVTQREEIDQLQEEMALILNNHIMEQQS-RL----
CAR_rat NFFCGP---LCYKMEDAVH----VGFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPDRPGVTQREEIDQLQEEVALILNNHIMEQQS-RL----
CAR_rhesus NFLCGP---LRYTIEDAARVSPAVGFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSPDRPGVTQRHEIDQLQEEMALTLQSYIKGQQQ-RP----
CAR_chimpanzee NFLCGP---LRYTIEDGAR----VGFQVEFLELLFHFHGTLRKLQLQEPEYVLLAAMALFSPDRPGVTQRDEIDQLQEEMALTLQSYIKGQQR-RP----
CAR_cow NFLCGP---LRYTIEDAAQ----AGFQEEFLEFLFGFHRTLRQLQLQEPEYVLMAAMALFSPDRPGITCREEIDQLQEEMALTLQNYIQGQQP-RP----
CAR_opossum TFLCGP---LRYTFQDGAH----VGFQEHFLELLLRFHMTLRRLKLQEPEYVLMAALALFSPDRPGVTQREQIDQFQEEMALTLQNYIRSQQA-RP----
CAR_fur_seal NFLCGP---LCYALEDGVH----VGFQEEFLELLFRFHATLRRLQLQEPEYVLMAAMALFSPDRPGVTRREEIDRLQEVTALTLQSYIKGQPP-RP----
CAR_baikal_seal NFLCGP---LRYTLEDGVH----VGFQEEFLELLFRFHATLRRFQLQEPEYVLMAAMALFSPDRPGVTQKEEIDRLQEMMALTLQSYIKGQPP-RH----
CAR_dog HFLCGP---LRYTMEDGVHGLSPAGFQEEFLELLFRFHGTLKRLQLQEPEYVLLAAMALFSPDRPGVTRREEIDHLQEVMALTLQSYIRGQQP-RP----
CAR_pig KFLCGP---LRYTIEDGAH----VGFQEEFLELLFGFHKTLRRLQLQEPEYVLMVAVALFSPDRPGVTQRKEIDQLQEEMALTLQSYIKGQQP-SL----
EcR_tick SIVFAN--NQPYTRDNYRS-----ASVGDSADALFRFCRKMCQLRVDNAEYALLTAIVIFS-ERPSLVDPHKVERIQEYYIETLRMYSENHRPPGK----
EcR_silkworm SVLFAN--NKAYTRDNYRQ-----GGMAYVIEDLLHFCRCMFAMGMDNVHFALLTAIVIFS-DRPGLEQPSLVEEIQRYYLNTLRIYIINQNSASSR---
EcR/FXR_sea_urchin AIMFGN--EMPYTRKQLLE-----GGIGDLVDPMYNFAKSMSELDLDYAEFILLMAITILSPDRPAINERERVEQMQETYLDMLRSYLKLRRPHEV----

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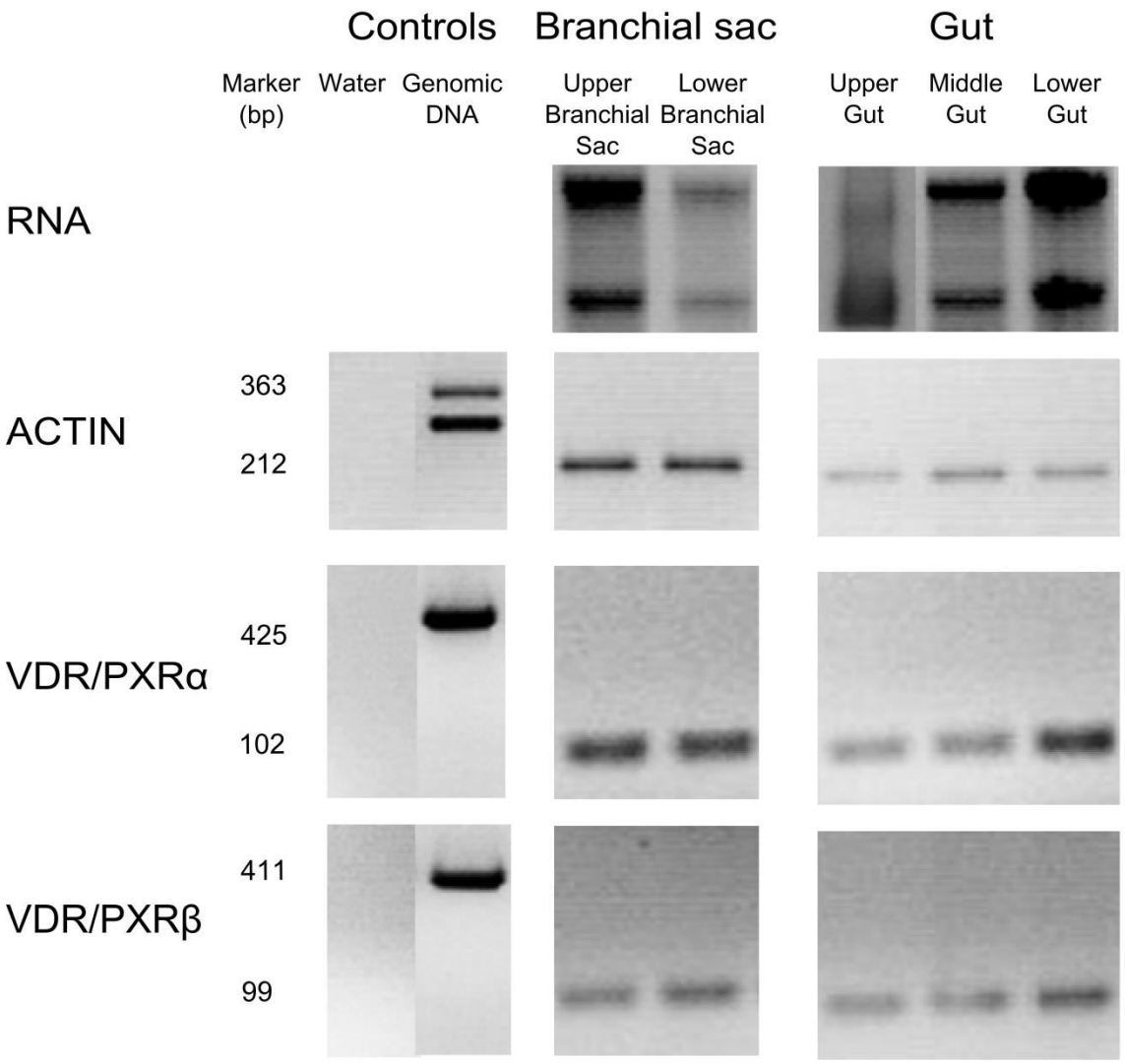
                                 210       220       230       240
                          ....|....|....|....|....|....|....|....|....|..
VDR_human                 GSHLLYAKMIQKLADLRSLNEEHSKQYRCLSFQPECSMKLTPLVLEV
VDR_mouse                 GSHQLYAKMIQKLADLRSLNEEHSKQYRSLSFQPENSMKLTPLVLEV
VDR_rhesus                GSHLLYAKMIQKLADLRSLNEEHSKQYRCLSFQPECSMKLTPLVLEV
VDR_chimpanzee            GSHLLYAKMI----------------------------------------
VDR_cow                   GSHLLYAKMIQKLADLRSLNEEHSKQYRCLSFQPESSMKLTPLLFEV
VDR_rat                   GSHQLYAKMIQKLADLRSLNEEHSKQYRSLSFQPENSMKLTPLVLEV
VDR_xenopus_laevis        GSRLLYAKMIQKLADLRSLNEEHSKQYRSISFLPEHSMKLTPLMLEV
VDR_stickleback           GGRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEV
VDR_medaka                GGRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEV
VDR_zebrafish             GGRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEV
VDR_lamprey               CKHMLYTKMVEKLTELRSLNEEHSKQYLQISQDAVNKEDLPPLLLEV
VDR_quail                 GSRLLYAKMIQKLADLRSLNEEHSKQYRCLSFQPEHSMQLTPLVLEV
VDR_halibut               GGRLLYAKMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEV
VDR_salmon                GGRLLYARMIQKLADLRSLNEEHSKQYRSLSFQPEHSMQLTPLVLEV
PXR_human                 AHRFLFLKIMAMLTELRSINAQHTQRLLRIQDIHP---FATPLMQEL
PXR_mouse                 AHRFLFLKIMAVLTELRSINAQQTQQLLRIQDSHP---FATPLMQEL
PXR_rat                   AHRFLFLKIMAVLTELRSINAQQTQQLLRIQDTHP---FATPLMQEL
PXR_rabbit                THRFLFLKIMAVLTELRTINAQHTQRLLRIQDTHP---FATPLMREL
PXR_chicken               EGRFLYAKLLLLLTELQTLKMENTRQILHIQDLSS----MTPLLSEI
PXR_rhesus                AHRFLFLKIMAMLTELRSINAQHTQRLLRIQDIHP---FATPLMQEL
PXR_medaka                GKHLLYPKIIACLTELRSMSEEHSKQILQIQDIQPD--TITPLLMEV
PXR_xenopus_tropicalis    QNRLLYPKIMECLTELRTVNDIHSKQLLEIWDIQP---DATPLLREV
BXRa_xenopus              QNRLLYPKIMECLTELRTVNDIHSKQLLEIWDIQP---DATPLMREV
BXRb_xenopus              QNRLLYPKIMECLTELRTINDIHSKQLMEIWDIQP---DVTPLMREV
PXR_zebrafish             EKHLLFPKIMGCLTEMRSMNEEYTKQVLKIQDMQP---EVSPLWLEI
PXR_opossum               AHRFLFLKIIAILTELRTINAQHTKRLLQIEDIHP---FATPLMREL
PXR_cow                   AHRFLFLKIMAILTELRSLSAEHTQQLLRIHDVHP---FATPLMQEL
PXR_chimpanzee            AHRFLFLKIMAMLTELRSINAQHTQRLLRIQDIHP---FATPLMQEL
VDR/PXRa_ciona_(NP_001071847)  -VRNRFAIIMSFLPRLRTLNSLCTTAFSQVK-KQFGE-EIRPLVKEV
VDR/PXRb_ciona_(NP_001037831)  QARTMYCELLLILPILRTINMLFAQNIMSLKQTNEK--DMNPLILEV
VDR/PXRa_ciona_(KC561370)      -IRNRFAIIMSFLPRLRTLNSLCTTAFSQVK-KQFGE-EIR------
VDR/PXRb_ciona_(KC561371)      -ARTMYCELLLILPILRTIN----------------------------
VDR/PXRa_botrullus_(KC561372)  -SRVRFPKIMAYLTRLRTLNWHISKTLDRIQSTSEAN-DIKPL----
CAR_human                 RDRFLYAKLLGLLAELRSINEAYGYQIQHIQGLSA----MMPLLQEI
CAR_mouse                 QSRFLYAKLMGLLADLRSINNAYSYELQRLEELSA----MTPLLGEI
CAR_rat                   QSRFLYAKLMGLLAELRSINSAYSYEIHRIQGLSA----MMPLLGEI
CAR_rhesus                RDRFLYAKLLGLLAELRSINEAYGYQIQHIQGLSA----MMPLLQEI
CAR_chimpanzee            RDRFLYAKLLGLLAELRSINEAYGYQIQHIQGLSA----MMPLLQEI
CAR_cow                   RDRFLYAKLLGLLADLRSIHDAFWYQIQNIQGLST----MMPLLQEI
CAR_opossum               QGRFLYAKMLGLLAELRSLSTEYGRQLQRIQELSA----LMPLLQEI
CAR_fur_seal              RDRFLYAKLLGLLAELRSIDNAYGYQIQHIQGLSA----MMPLLQEI
CAR_baikal_seal           RDRFLYAKLLGLLAELRSINNAYGYQIQHIQGLSA----MMPLLQEI
CAR_dog                   RDRFLYAKLLGLLAELRSINNAYGHQIQHIQGLSA----MMPLLQEI
CAR_pig                   RDRFLYAKLLGLLAELRSINKEYWYQIQNIQGLST----MMPLLQEI
EcR_tick                  ---NYFARLLSILTELRTLGNMNAEMCFSLKVQNK---KLPPFLAEI
EcR_silkworm              -CAVIYGRILSVLTELRTLGTQNSNMCISLKLKNR---KLPPFLEEI
EcR/FXR_sea_urchin        ---LLLPKVLMKLTELRSLNNSHSELLFQLKVKDQ---KIPPLLQEI
```

Appendix 3 Alignment of predicted DNA-binding domains (DBDs) and ligand-binding domains (LBDs) from pregnane X receptor (PXR)-related proteins from vertebrates, invertebrate chordates and protostome taxa. GenBank accession numbers are as follows: human (*Homo sapiens*) VDR [NM_00376], rhesus monkey (*Macaca mulatta*) VDR [Ensembl: ENSMMUT00000009414], cow (*Bos primigenius*) VDR [Ensembl: ENSBTAT00000021832], mouse (*Mus musculus*) VDR [NM_008504], rat (*Rattus norvegicus*) VDR [NM_009504], *Xenopus laevis* VDR [U91849], zebrafish (*Danio rerio*) VDR [AF164512], medaka (*Oryzias latipes*) VDR [Ensembl: ENSORLT00000001311], stickleback fish (*Gastrosteus aculeatus*) VDR [Ensembl: ENSGACT00000006308], sea lamprey (*Petromyzon marinus*) VDR [AY249863], quail (*Coturnix japonica*) VDR [AAA56725], halibut (*Paralichthys olivaceus*) VDR [BAA95016], salmon (*Salmo salar*) VDR [CAG47089], *Ciona intestinalis* VDR/PXRα [NP_001071847 and KC561370], *Ciona intestinalis* VDR/PXRβ [NP_001037831 and KC561371], *Botryllus schlosseri* VDR/PXRα [KC561372], human PXR [AF061056], rhesus monkey PXR [AF454671], mouse PXR [NP_035066], rat PXR [AF151377], rabbit (*Oryctolagus cuniculus*) PXR [AF31165], chicken (*Gallus gallus*) PXR [AF276753], *Xenopus laevis* BXRα [BC041187], *Xenopus laevis* BXRβ [AF305201], *Xenopus tropicalis* PXR [Ensembl: ENSXETT00000039109], medaka PXR [Ensembl: ENSORLT00000022473], zebrafish PXR [AF454674], opossum PXR [Ensembl: ENSMODT00000023109], cow (*Bos taurus*) PXR [Ensembl: ENSBTAT00000026059], chimpanzee (*Pan troglodytes*) PXR [Ensembl: ENSPTRT00000028510], human CAR [NP_005113], mouse CAR [NP_033933], rat CAR [NP_075230], rhesus monkey CAR [AAM76230], chimpanzee CAR [NP_001129087], cow CAR [NP_001073236], opossum CAR [Ensembl: ENSMODT00000006393], fur seal (*Callorhinus ursinus*) CAR [BAD00039], Baikal seal (*Phoca sibirica*) CAR [BAD00038], dog (*Canis sp.*) CAR [Ensembl: ENSCAFT00000020528], pig (*Sus scrofa*) CAR [BAE54304], domestic silkworm (*Bombyx mori*) EcR [AAA87341], tick (*Amblyomma americanum*) EcR [AAB94566] and sea urchin (*Strongylocentrotus purpuratus*) EcR/FXR [NP_001123279]. Alignment was modified from Ekins *et al*. (2008).

APPENDIX FOUR



Appendix 4 Amplification of *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (VDR/PXRα), VDR/PXRβ and actin partial coding sequences in five tissues using end-point polymerase chain reaction (PCR). Primers used for amplification are listed in Appendix 1B. *Abbreviations*: bp, base pair.

APPENDIX FIVE

Appendix 5 Approximate probabilities (p) of Cochran's C Test (**A**) and Tukey HSD Post Hoc Test (**B**) for the expression of *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ) and *C. intestinalis* actin (*Ci*actin) in various gut tissues. (**A**) Homogeneous data (non-significant Cochran C) is shown in black numbers and non-homogeneous data (significant Cochran C) is highlighted in red numbers. (**B**) P-values with $p < 0.01$ were considered statistically significant (red numbers).

A		**Hartley F-max**	**Cochran C**	**Bartlett Chi-Sq.**	**df**	**p**
	*Ci*VDR/PXRα	21.62	0.63	5.14	4	0.273
	*Ci*VDR/PXRβ	1312	0.91	21.8	4	0.001
	*Ci*actin	35.36	0.51	5.08	4	0.278

B	***Ci*VDR/PXRα**	**Upper Branchial Sac**	**Lower Branchial Sac**	**Upper Gut**	**Middle Gut**	**Lower Gut**
	Upper Branchial Sac					
	Lower Branchial Sac	0.42				
	Upper Gut	0.01	0.01			
	Middle Gut	0.01	0.01	0.01		
	Lower Gut	0.01	0.01	0.01	0.01	
	***Ci*VDR/PXRβ**					
	Upper Branchial Sac					
	Lower Branchial Sac	0.92				
	Upper Gut	0.37	0.80			
	Middle Gut	0.02	0.07	0.37		
	Lower Gut	0.01	0.00	0.01	0.01	
	***Ci*actin**					
	Upper Branchial Sac					
	Lower Branchial Sac	1.00				
	Upper Gut	0.01	0.01			
	Middle Gut	0.01	0.01	0.01		
	Lower Gut	0.01	0.01	0.01	0.01	

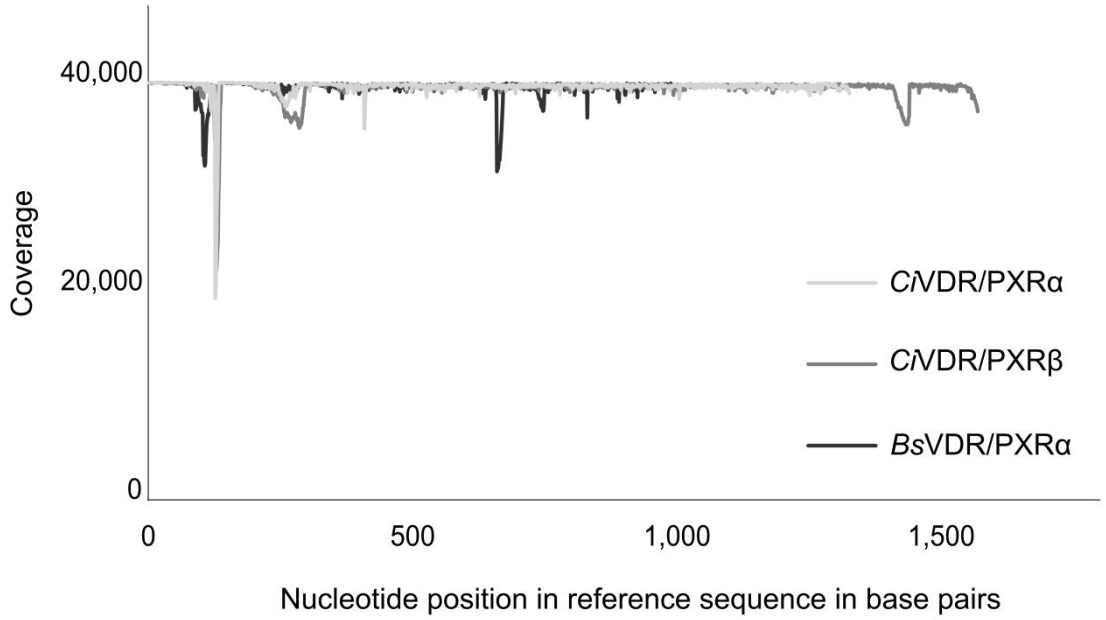
Abbreviations: *Ci*, *Ciona intestinalis*; *Bs*, *Botryllus schlosseri*; df, degrees of freedom.

APPENDIX SIX

Appendix 6 Illumina™ sequencing read mapping statistics for *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα, KC561370), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, KC561371) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα, KC561372).

	Reads mapping to reference sequences		
	Pair end	**Single end read 1**	**Single end read 2**
*Ci*VDR/PXRα	2,795,419 (36%)	1,380,943 (35%)	1,376,933 (35%)
*Ci*VDR/PXRβ	1,923,917 (24%)	944,154 (24%)	940,214 (24%)
*Bs*VDR/PXRα	2,892,225 (37%)	1,334,773 (35%)	1,363,318 (34%)
Unmapped	262,741 (3%)	277,283 (6%)	256,688 (7%)
Total	7,874,302	3,937,153	3,937,153

APPENDIX SEVEN



Appendix 7 Coverage by position for *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα, KC561370), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, KC561371) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα, KC561372) using a maximum coverage of 40,000.

APPENDIX EIGHT

Appendix 8 Nucleotide polymorphisms detected in *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα, 1326 base pairs, bp), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, 1569 bp) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα, 1006 bp) using Illumina™ sequencing. Number of single nucleotide polymorphisms (SNPs) and indels are given for various minimum variant frequencies ($f_{min\ var}$). Shaded area indicates the minimum variant frequency 'cut-off' value (0.02) used in subsequent analyses.

	***Ci*VDR/PXRα**			***Ci*VDR/PXRβ**			***Bs*VDR/PXRα**			**Total**	
$f_{min\ var}$	T_{SNPs}	f_{SNPs}	T_{indel}	T_{SNPs}	f_{SNPs}	T_{indel}	T_{SNPs}	f_{SNPs}	T_{indel}	T_{SNPs}	T_{indel}
0.01	28	2.11	2	53	3.38	5	42	4.17	1	42	3
0.02	28	2.11	1	44	2.81	1	39	3.88		38	1
0.03	26	1.96	1	43	2.74		38	3.78		36	1
0.04	26	1.96	1	40	2.55		37	3.68		35	1
0.05	25	1.88	1	39	2.48		37	3.68		34	1
0.06	25	1.88	1	38	2.55		37	3.68		34	1
0.07	21	1.58	1	36	2.42		37	3.68		32	1
0.08	19	1.43	1	34	2.29		36	3.58		30	1
0.09	17	1.28		32	2.17		36	3.58		29	
0.1	16	1.21		31	1.97		35	3.48		28	
0.11	16	1.21		30	1.91		34	3.38		27	
0.12	16	1.21		29	1.85		34	3.38		27	
0.13	16	1.21		29	1.85		34	3.38		27	
0.14	15	1.13		26	1.66		34	3.38		26	
0.15	15	1.13		26	1.66		31	3.08		25	
0.16	15	1.13		24	1.53		31	3.08		24	
0.17	15	1.13		24	1.53		30	2.98		24	
0.18	15	1.13		22	1.40		29	2.88		23	
0.19	14	1.06		19	1.21		27	2.68		21	
0.2	14	1.06		19	1.21		27	2.68		21	

Abbreviations: $f_{min\ var}$, minimum variant frequency 'cut-off' values; T_{SNPs}, total SNPs; f_{SNPs}, SNP frequency (SNP/100 bp); T_{indel}, total one base pair indels.

APPENDIX NINE

Appendix 9 Estimated transition/transversion rate ratios (Ti/Tv) for *Ciona intestinalis* vitamin D receptor/pregnane X receptor α (*Ci*VDR/PXRα, 1326 base pairs, bp), *C. intestinalis* VDR/PXRβ (*Ci*VDR/PXRβ, 1569 bp) and *Botryllus schlosseri* VDR/PXRα (*Bs*VDR/PXRα, 1006 bp) using the Tamura-Nei model as implemented in the MEGA 5 software package.

Substi-	**Counts**			**Percent of total counts**			**Rate of individual event**		
tution	***Ci*VDR/PXRα**	***Ci*VDR/PXRβ**	***Bs*VDR/PXRα**	***Ci*VDR/PXRα**	***Ci*VDR/PXRβ**	***Bs*VDR/PXRα**	***Ci*VDR/PXRα**	***Ci*VDR/PXRβ**	***Bs*VDR/PXRα**
Transitions									
All	22	31	31	78	71	80	0.017	0.022	0.032
A -> G	9	14	17	32	32	44			
T -> C	13	17	14	46	39	36			
Transversions									
All	6	13	8	22	29	20	0.005	0.009	0.008
A -> T	1	4	6	3.5	9	15			
A -> C	2	3	1	7.5	7	2.5			
T -> G	2	4	1	7.5	9	2.5			
C -> G	1	2	0	3.5	4	0			
Ti/Tv ratios							**3.7**	**2.4**	**3.9**

APPENDIX TEN

Appendix 10 Nucleotide polymorphisms detected in *Botryllus schlosseri* vitamin D receptor/pregnane X receptor α (*Bs*VDR/PXRα, 1006 base pairs, bp) populations from Nelson Marina (New Zealand) and the eastern Mediterranean Coast (Michmoret Beach, Israel). One sequence from each population was selected as a reference sequence. Shaded areas indicate single nucleotide polymorphisms (SNPs) that were detected in a minimum of two sequences from independent amplification reactions.

Position	***Bs*VDR/PXRα Nelson Marina**		
(bp)	**Ref**	**Var**	**aa subst.**
9	T	C	M -> T
11	G	A	K -> E
32	T	C	C -> R
40	T	C	
41	A	G	T -> A
59	A	G	T -> A
61	G	A	
76	A	G	
80	T	C	C -> R
82	T	C	
86	G	A	A -> T
94	G	A	
95	T	C	
108	T	C	L -> P
115	T	C	
130	G	A	
135	C	T	I -> T
144	G	A	H -> R
148	G	A	
154	T	C	
162	A	G	K -> R
181	C	T	
194	C	G	R -> G
197	T	A	S -> C
199	T	C	
209	T	C	W -> R
218	T	C	
246	T	C	L -> P
252	C	T	T -> M
259	C	A	
271	T	C	
281	A	G	N -> D
303	A	G	E -> G
352	G	A	
361	A	G	
405	T	G	I -> S
421	G	A	

Position	***Bs*VDR/PXRα Israel**		
(bp)	**Ref**	**Var**	**aa subst.**
8	A	G	M -> V
14	A	G	N -> D
31	T	A	
40	C	T	
49	T	C	
57	T	C	I -> T
60	C	T	T -> M
61	G	A	
65	A	G	S -> G
76	A	G	
82	C	T	
85	A	G	
95	C	T	
106	C	T	
115	C	T	
134	A	G	I -> V
144	A	G	H -> R
145	C	T	
148	A	G	
152	A	G	I -> V
169	T	C	
180	A	G	N -> S
197	A	T	S -> C
199	T	C	S -> C
213	A	G	K -> R
252	C	T	T -> M
260	T	C	S -> P
271	C	T	
281	A	G	N -> D
289	T	A	
303	A	G	E -> G
305	G	A	E -> K
309	A	G	D -> G
333	G	T	R -> I
349	C	T	
361	A	G	
369	G	A	G -> E

Appendix 10 (cont.) Nucleotide polymorphisms detected in *Botryllus schlosseri* vitamin D receptor/pregnane X receptor α (*Bs*VDR/PXRα, 1006 base pairs, bp) populations from Nelson Marina (New Zealand) and the eastern Mediterranean Coast (Michmoret Beach, Israel).

Position	*Bs*VDR/PXRα Nelson Marina		
(bp)	Ref	Var	(bp)
424	A	G	
456	A	G	H -> R
460	T	C	
489	A	G	E -> G
517	T	C	
553	G	A	
576	A	G	E -> G
580	A	C	
607	C	T	
625	A	T	
643	G	A	
658	G	A	
661	A	T	D -> E
667	T	C	
686	G	A	E -> K
689	G	A	E -> K
703	C	T	
709	G	A	
733	T	C	
734	A	G	M -> V
742	T	C	
748	A	G	
760	G	T	
763	C	T	
774	C	T	T -> I
775	A	G	
781	G	A	
815	A	T	S -> T
822	T	C	V -> A
865	C	T	
896	T	C	F -> L
903	A	G	K -> R
913	G	A	
919	G	A	
945	A	G	H -> R
953	A	G	K -> E
954	A	G	
999	A	G	K -> R
1000	G	A	

Position	*Bs*VDR/PXRα Israel		
(bp)	Ref	Var	aa subst.
374	T	C	S -> P
387	A	G	N -> S
404	A	G	
405	T	C	I -> V or T
406	C	T	
409	A	G	
421	G	A	
424	G	A	
426	C	T	P -> L
443	T	C	S -> P
447	A	G	H -> R
465	C	T	A -> V
475	G	A	
491	T	C	
536	T	C	
553	G	A	
569	T	C	C -> R
573	C	T	T -> M
580	C	A	
619	G	A	
625	T	A	
627	T	C	L -> S
661	T	A	D -> E
667	C	T	
669	T	C	I -> T
673	A	G	
680	A	G	M -> V
687	A	G	E -> G
703	C	T	
715	T	C	
726	T	C	L -> P
733	T	C	
737	C	T	
748	G	A	
760	T	G	
763	T	C	
765	T	C	M -> T
772	C	T	
775	G	A	
781	G	A	
787	T	C	
800	G	- G	
819	T	C	L -> P
826	A	G	
835	G	C	Q -> H
837	A	G	E -> G
853	T	C	
865	C	T	
896	T	C	F -> L

Appendix 10 (cont.) Nucleotide polymorphisms detected in *Botryllus schlosseri* vitamin D receptor/pregnane X receptor α (*Bs*VDR/PXRα, 1006 base pairs, bp) populations from Nelson Marina (New Zealand) and the eastern Mediterranean Coast (Michmoret Beach, Israel).

Position	***Bs*VDR/PXRα Nelson Marina**			**Position**	***Bs*VDR/PXRα Israel**		
(bp)	**Ref**	**Var**	**(bp)**	**(bp)**	**Ref**	**Var**	**aa subst.**
				919	G	A	
				931	A	G	
				991	C	T	
				1000	A	G	
T_{SNPs}		76	34 (44%)			89	42 (47%)
f_{SNPs}		7.55	3.38			8.85	3.97

Abbreviations: aa subst., amino acid substitution caused by SNP in variant sequence; bp, base pair; f_{SNPs}, SNP frequency (SNP/100 bp); Ref, reference sequence; T_{SNPs}, total SNPs; Var, variant sequence.

APPENDIX ELEVEN

Appendix 11 Activation of the tunicate yeast bioassays by four synthetic chemicals (**A**) and five algal toxins (**B**). Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in µM for synthetic chemicals (**A**) and in nM for algal toxins (**B**). 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in dimethyl sulfoxide (DMSO).

A

Chemical name	**Toxin type**	**Supplier (Catalogue No.)**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
			EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
N-butyl-*p*-amino-benzoate [a]	Local anaesthetic	Sigma-Aldrich (B7753)	9.4 (7.8–11)	25	6.1 (4.9–7.5)	10	2.4 (0.5–12)	28
Carbamazepine [b]	Anti-depressant	Sigma-Aldrich (C4024)	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC
P-aminobenzoic acid [b]	Supplement	Sigma-Aldrich (A9878)	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC
Bisphenol-A (BPA) [b]	EDC	Sigma-Aldrich (B1760)	76 (67–85)	11	66 (59–73)	17	58 (48–69)	16

Appendix 11 (cont.) Activation of the tunicate yeast bioassays by four synthetic chemicals (**A**) and five algal toxins (**B**).

B

Chemical name	**Toxin type**	**Supplier (Catalogue No.)**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
			EC_{50} (95% CI)	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Okadaic acid [b]	Microalgal biotoxin	Sapphire Bioscience (AB120375)	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC
Pectenotoxin-11 [b]	Microalgal biotoxin	MacKenzie *et al.* 2013	913 (238–3506)	154	545 (150–1985))	63	NI (NI)	DNC
Portimine [b]	Microalgal biotoxin	Selwood *et al.* 2013	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC
Microcystin-RR [b]	Cyanobac-terial toxin	DHI Lab Products (PPS-MCRR)	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC
Anatoxin-A [b]	Cyanobac-terial toxin	NRC Canada (IMB-CRM-ATX)	NI (NI)	DNC	NI (NI)	DNC	NI (NI)	DNC

Abbreviations: BPA, bisphenol-A; DNC, did not compute; EDC, endocrine disrupting chemical; NI, no induction of β-galactosidase enzymatic activity.

[a] Strains were incubated for 24 hours.

[b] Strains were incubated for 48 hours.

APPENDIX TWELVE

Appendix 12 Summary of the templates, primers and restriction enzymes used to generate the plasmids used in this study. Sites for restriction enzymes encoded within the primer sequences are underlined and regions annealing to target genes are indicated in grey. The base vector pGAL4 was obtained from Clontech (Clontech Laboratories Inc., Catalogue No. 630443).

Plasmid	Template	Primers	Region amplified	Enzymes	Base vector
pGAL4	N/A	N/A	N/A	N/A	pGBKT7
pGAL4.VP16	pHCA/GAL4(1–93).ER.VP16 [a]	forward: 5'–CCGGATCCGTGAGCTCCACTTAGACGGCGAGGAC–3' reverse: 5'–CCCTGCAGCTACCCACCGTACTCGTCAATTC–3'	103,274–103,480 of HM585511	BamHI/ PstI	pGBKT7
pGAL4.*Ci*LBD	pM2-GAL4-*Ci*VDR/PXRα [b]	forward: 5'–CCCCATGGAGGGAATGAAGCGCGAATGTATCATGTCA–3' reverse: 5'–CCGGATCCCATTCAAGCGTTTTCCACGG–3'	860–1798 of NM_001078379	NcoI/ BamHI	pGBKT7
pGAL4.*Ci*LBD .VP16	pM2-GAL4-*Ci*VDR/PXRα [b]	forward: 5'–CCCCATGGAGGGAATGAAGCGCGAATGTATCATGTCA–3' reverse: 5'–CCGGATCCCAGCGTTTTCCACGGTGGGTTGAAC–3'	860–1795 of NM_001078379	NcoI/ BamHI	GAL4.VP16

Appendix 12 (cont.) Summary of the templates, primers and restriction enzymes used to generate the plasmids used in this study.

Plasmid	Template	Primers	Region amplified	Enzymes	Base vector
pGAL4.*Ci*LBD Δ31.VP16	pM2-GAL4-*Ci*VDR/PXRα [b]	forward: 5'–CCCCATGGAGGGAATGAAGCGCGAATGTATCATGTCA–3' reverse: 5'–CCGGATCCCACTGTTGAGGGTTCTCAGCCTTGG–3'	860–1702 of NM_001078379	NcoI/ BamHI	GAL4.VP16
pGAL4.*Bs*LBD .VP16	*Botryllus schlosseri* cDNA	forward: 5'–CCCCATGGAGGGCATGAAGAAAGAGTGCATCATG–3' reverse: 5'–CCGGATCCCCACGTTGGGCATACATTCAAATAC–3'	116–1006 [c] of KC561372	NcoI/ BamHI	GAL4.VP16
pGAL4.*Bs*LBD	*Botryllus schlosseri* cDNA	forward: 5'–CCCCATGGAGGGCATGAAGAAAGAGTGCATCATG–3' reverse: 5'–CCGGATCCTCATTACACGTTGGGCATACATTCAAATAC–3'	116–1006 [c] of KC561372	NcoI/ BamHI	GAL4.VP16

Abbreviations: N/A, not available.

[a] Plasmid pHCA/GAL4(1–93).ER.VP16 was generously provided by Prof Didier Picard, University of Geneva, Switzerland (Louvion *et al.* 1993).

[b] Plasmid pM2-GAL4-*Ci*VDR/PXRα was generously provided by Prof Matthew Krasowski, University of Iowa, U.S.A. (Reschly *et al.* 2007; Ekins *et al.* 2008; Fidler *et al.* 2012).

[c] Reverse primer positioned immediately 3' to the KC561372 sequence.

APPENDIX THIRTEEN

Appendix 13 Summary of the predicted proteins encoded by the seven plasmids used in this study. Stop codons are indicated (*).

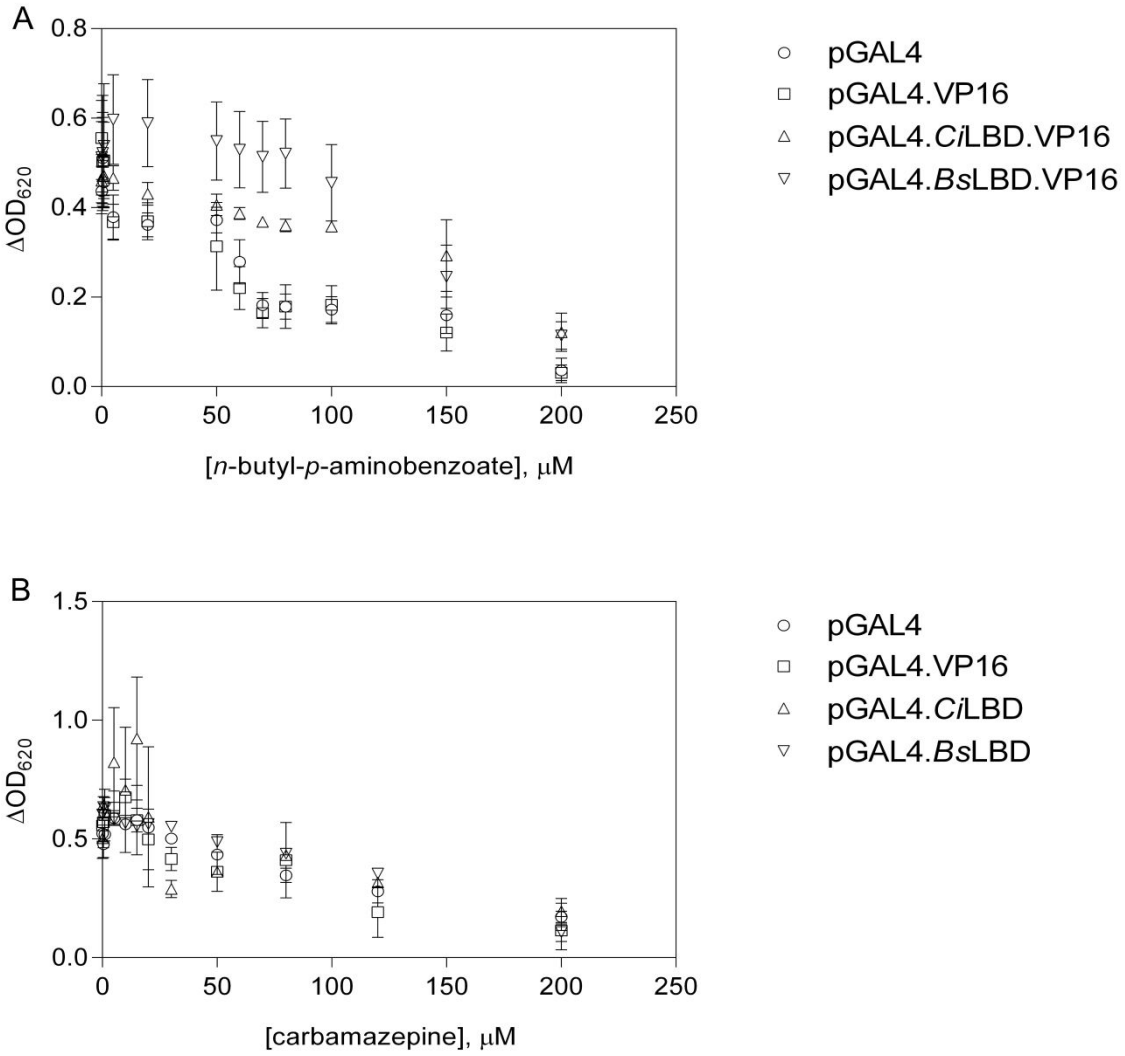
Plasmid name	**GAL4-DBD sequence (147 residues)**	**Linker sequence**	**LBD sequence**	**C-terminal/ VP16 sequence**
pGAL4	MKLLSSIEQACDICRLKKLKCSKEK PKCAKCLKNNWECRYSPKTKRSPL TRAHLTEVESRLERLEQLFLLIFPRE DLDMILKMDSLQDIKALLTGLFVQD NVNKDAVTDRLASVETDMPLTLRQ HRISATSSSEESSNKGQRQLTVS	PEFVIRLTI GRAAIMEE QKLISEED LHMAME	N/A	AEFPGIRRPAA AA*
pGAL4.VP16	MKLLSSIEQACDICRLKKLKCSKEK PKCAKCLKNNWECRYSPKTKRSPL TRAHLTEVESRLERLEQLFLLIFPRE DLDMILKMDSLQDIKALLTGLFVQD NVNKDAVTDRLASVETDMPLTLRQ HRISATSSSEESSNKGQRQLTVS	PEFVIRLTI GRAAIMEE QKLISEED LHMAMEA EFP	N/A	GIRELHLDGED VAMAHADALD DFDLDMLGDG DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG*
pGAL4.*Ci*LBD	MKLLSSIEQACDICRLKKLKCSKEK PKCAKCLKNNWECRYSPKTKRSPL TRAHLTEVESRLERLEQLFLLIFPRE DLDMILKMDSLQDIKALLTGLFVQD NVNKDAVTDRLASVETDMPLTLRQ HRISATSSSEESSNKGQRQLTVS	PEFVIRLTI GRAAIMEE QKLISEED LHMAME	GMKRECIMSPDEIQMKKTLVLSNRIKRATMQWVPMELTN DQKILLDTICSAFIQSNNLPEKGVIRGGEEVRQDADKGSP CSSPSSSSNNSNFLELIKRIANDIAARTPSNMTNPAHVVHF TDIMEFSIKEIIKFCKKIPTFMDLDLKDQIALLKSGCTEILFIK ANYTYDLEKKALTLGPDILYTRDSFLQGGMSVEYTDNYLK FHEDLSALQLDDVEMSSLSAIALFSADRADLVDQQRVEN QQEALALCLQAYSESSWKVRNRFAIIMSFLPRLRTLNSLC TTAFSQVKKQFGEEIRPLVKEVQPTVENA*	N/A

Appendix 13 (cont.) Summary of the predicted proteins encoded by the seven plasmids used in this study.

pGAL4.*Ci*LBD.VP16	MKLLSSIEQACDICRLKKLKCSKE KPKCAKCLKNNWECRYSPKTKRS PLTRAHLTEVESRLERLEQLFLLIF PREDLDMILKMDSLQDIKALLTGL FVQDNVNKDAVTDRLASVETDMP LTLRQHRISATSSSEESSNKGQR QLTVS	PEFVIR LTIGRA AIMEE QKLISE EDLHM AME	GMKRECIMSPDEIQMKKTLVLSNRIKRATMQWVPMELTND QKILLDTICSAFIQSNNLPEKGVIRGGEEVRQDADKGSPCS SPSSSSNNSNFLELIKRIANDIAARTPSNMTNPAHVVHFTDI MEFSIKEIIKFCKKIPTFMDLDLKDQIALLKSGCTEILFIKANYT YDLEKKALTLGPDILYTRDSFLQGGMSVEYTDNYLKFHEDL SALQLDDVEMSSLSAIALFSADRADLVDQQRVENQQEALA LCLQAYSESSWKVRNRFAIIMSFLPRLRTLNSLCTTAFSQV KKQFGEEIRPLVKEVQPTVENA	GIRELHLDGED VAMAHADALD DFDLDMLGDG DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG*
pGAL4.*Ci*LBDΔ31.VP16	MKLLSSIEQACDICRLKKLKCSKE KPKCAKCLKNNWECRYSPKTKRS PLTRAHLTEVESRLERLEQLFLLIF PREDLDMILKMDSLQDIKALLTGL FVQDNVNKDAVTDRLASVETDMP LTLRQHRISATSSSEESSNKGQR QLTVS	PEFVIR LTIGRA AIMEE QKLISE EDLHM AME	GMKRECIMSPDEIQMKKTLVLSNRIKRATMQWVPMELTND QKILLDTICSAFIQSNNLPEKGVIRGGEEVRQDADKGSPCS SPSSSSNNSNFLELIKRIANDIAARTPSNMTNPAHVVHFTDI MEFSIKEIIKFCKKIPTFMDLDLKDQIALLKSGCTEILFIKANYT YDLEKKALTLGPDILYTRDSFLQGGMSVEYTDNYLKFHEDL SALQLDDVEMSSLSAIALFSADRADLVDQQRVENQQEALA LCLQAYSESSWKVRNRFAIIMSFLPRLRTLNS	GIRELHLDGED VAMAHADALD DFDLDMLGDG DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG*
pGAL4.*Bs*LBD	MKLLSSIEQACDICRLKKLKCSKE KPKCAKCLKNNWECRYSPKTKRS PLTRAHLTEVESRLERLEQLFLLIF PREDLDMILKMDSLQDIKALLTGL FVQDNVNKDAVTDRLASVETDMP LTLRQHRISATSSSEESSNKGQR QLTVS	PEFVIR LTIGRA AIMEE QKLISE EDLHM AME	GMKKECIMSHTEIQMKKNLMLNNKIKRSVMEWKPLEYSAE RRQLVTLISESHTQTNGPPDIREEDGMLTIRSRDPGASPAS TSSGFSDLINQMTQDIVATAPGPSNPSKSHLRHFSAIMEFSI KEIIKFCKKIPSFTSLSLKDQITLLKSGCTEILFIKANYTYDRE QNALMCGPGKYYTRDSFILGGMSEEYTDCYLQFHHDLSH MLLDESELACMCATSLFSGDRDGLENRSLVEEVQERITVAL QSYTETIYHSRVRFPKIMAYLTRLRTLNWHISKTLDRIQSTS EANDIKPLVFECMPNV**	N/A
pGAL4.*Bs*LBD.VP16	MKLLSSIEQACDICRLKKLKCSKE KPKCAKCLKNNWECRYSPKTKRS PLTRAHLTEVESRLERLEQLFLLIF PREDLDMILKMDSLQDIKALLTGL FVQDNVNKDAVTDRLASVETDMP LTLRQHRISATSSSEESSNKGQR QLTVS	PEFVIR LTIGRA AIMEE QKLISE EDLHM AME	GMKKECIMSHTEIQMKKNLMLNNKIKRSVMEWKPLEYSAE RRQLVTLISESHTQTNGPPDIREEDGMLTIRSRDPGASPAS TSSGFSDLINQMTQDIVATAPGPSNPSKSHLRHFSAIMEFSI KEIIKFCKKIPSFTSLSLKDQITLLKSGCTEILFIKANYTYDRE QNALMCGPGKYYTRDSFILGGMSEEYTDCYLQFHHDLSH MLLDESELACMCATSLFSGDRDGLENRSLVEEVQERITVAL QSYTETIYHSRVRFPKIMAYLTRLRTLNWHISKTLDRIQSTS EANDIKPLVFECMPNV	GIRELHLDGED VAMAHADALD DFDLDMLGDG DSPGPGFTPH DSAPYGALDM ADFEFEQMFT DALGIDEYGG*

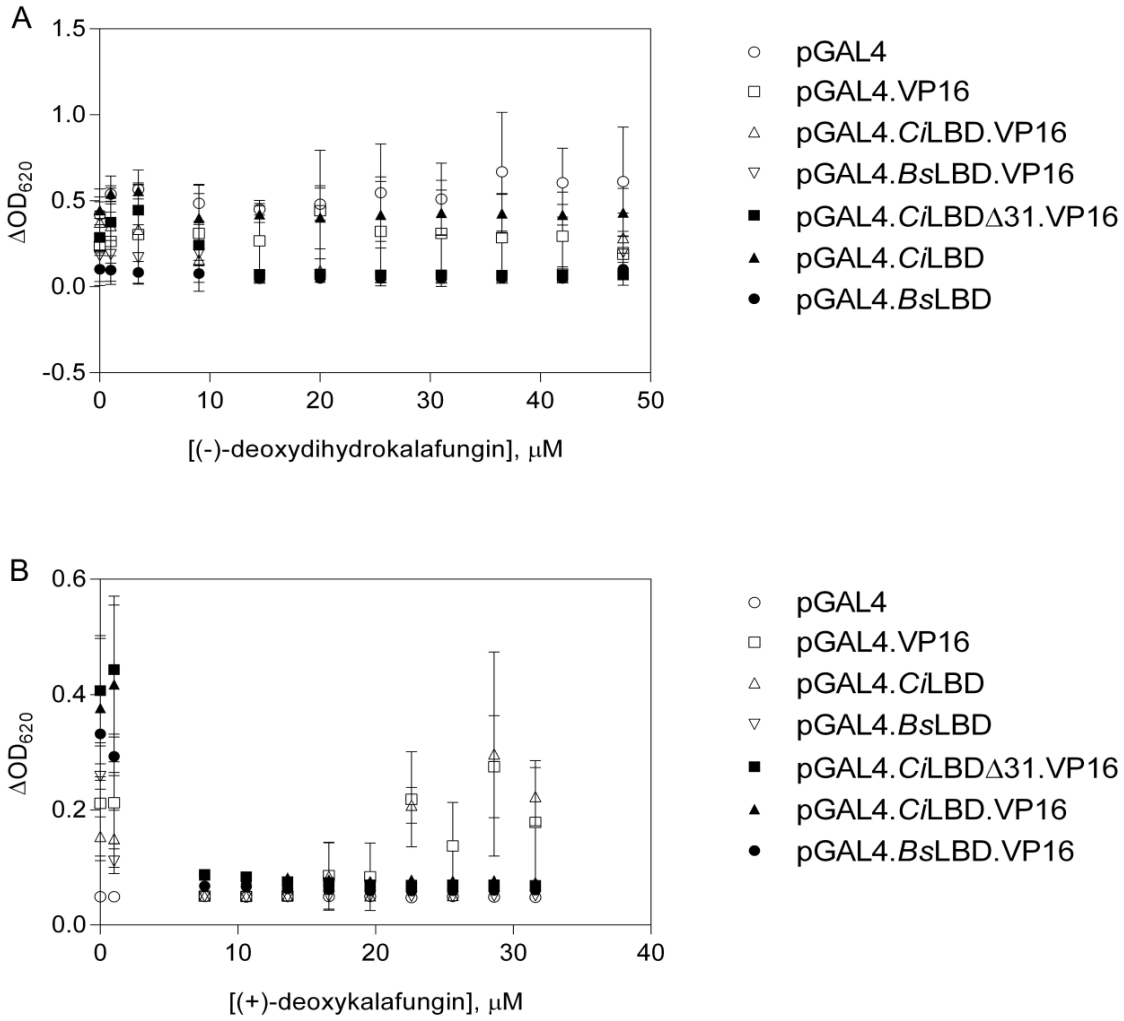
Abbreviations: N/A, not available.

APPENDIX FOURTEEN



Appendix 14 Suppression of growth of recombinant yeast strains by: (**A**) *n*-butyl-*p*-aminobenzoate and (**B**) carbamazepine. Cell densities were quantified by OD_{620} values. Growth suppression (i.e. toxic) effects were detected by changes in OD_{620} values over time (ΔOD_{620} = OD_{620} after 48 h – OD_{620} after 16 h). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 and pGAL4.*Bs*LBD.VP16 were omitted from this analysis because the media colouration arising from hydrolysis of chlorophenol red-β-D-galactopyranoside (CPRG) interfered with OD_{620} measurements.

APPENDIX FIFTEEN



Appendix 15 Suppression of growth of recombinant yeast strains by: (**A**) (-)-deoxydihydrokalafungin and (**B**) (+)-deoxykalafungin. Cell densities were quantified by OD_{620} values. Growth suppression (i.e. toxic) effects were detected by changes in OD_{620} values over time (ΔOD_{620} = OD_{620} after 24 h – OD_{620} after 16 h). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation.

APPENDIX SIXTEEN

Appendix 16 Activation of the yeast bioassays by 13 emerging contaminants.

Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in µM. 95% confidence intervals represent variation within a triplicate measurement (n = 3). Coefficients of variance (CV) are given in % for triplicate intra-plate measurements. All compounds were dissolved in dimethyl sulfoxide (DMSO).

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Butylated hydroxytoluene [a]	Antioxidant	Sigma-Aldrich (B1378)	7.1 (2.3–22)	37	7.2 (3.1–17)	41	NI (DNC)	DNC
4-chloro-3,5-dimethylphenol [b]	Antimicrobial	Sigma-Aldrich (C-4394)	15 (4.8–43)	39	61 (37–98)	41	29 (16–50)	25
2-phenoxyethanol [b]	Antimicrobial	Sigma-Aldrich (77699)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Propyl-4-hydroxy-benzoate [b]	Antimicrobial	Sigma-Aldrich (P53357)	237 (132–425)	34	115 (DNC)	39	NI (DNC)	DNC

Appendix 16 (cont.) Activation of the yeast bioassays by 13 emerging contaminants.

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
4-methyl-benzylidene camphor [b]	EDC	Sigma-Aldrich (78551)	NI (DNC)	DNC	101 (78–131)	34	103 (75–141)	36
Octyl methoxy-cinnamate [b]	EDC	Sigma-Aldrich (78848)	37 (20–67)	17	44 (13–151)	10	377 (2.4–60,283)	123
Triclosan [a]	EDC	Sigma-Aldrich (72779)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Benzophenone [b]	EDC	Sigma-Aldrich (B9300)	~382 (DNC)	DNC	348 (312–388)	23	NI (DNC)	DNC
Ketoconazole [a]	Antifungal	EMD Chemicals (420600)	~596 (DNC)[c]	DNC	509 (455–570)[c]	13	582 (391–865)[c]	18
2-phenylphenol [b]	Antifungal	Acros Organics (130760050)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
Radicicol [b]	Antifungal	Cayman Chemical (13089)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC
DEET [b]	Insecticide	Fluka (36542)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC

Appendix 16 (cont.) Activation of the yeast bioassays by 13 emerging contaminants.

Chemical name	**Toxin type**	**Supplier**	**pGAL4.*Ci*LBDΔ31.VP16**		**pGAL4.*Ci*LBD.VP16**		**pGAL4.*Bs*LBD.VP16**	
		(Catalogue No.)	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**	**EC_{50} (95% CI)**	**CV**
Diclofenac sodium salt [b]	Anti-inflammatory	Sigma-Aldrich (06899)	NI (DNC)	DNC	NI (DNC)	DNC	NI (DNC)	DNC

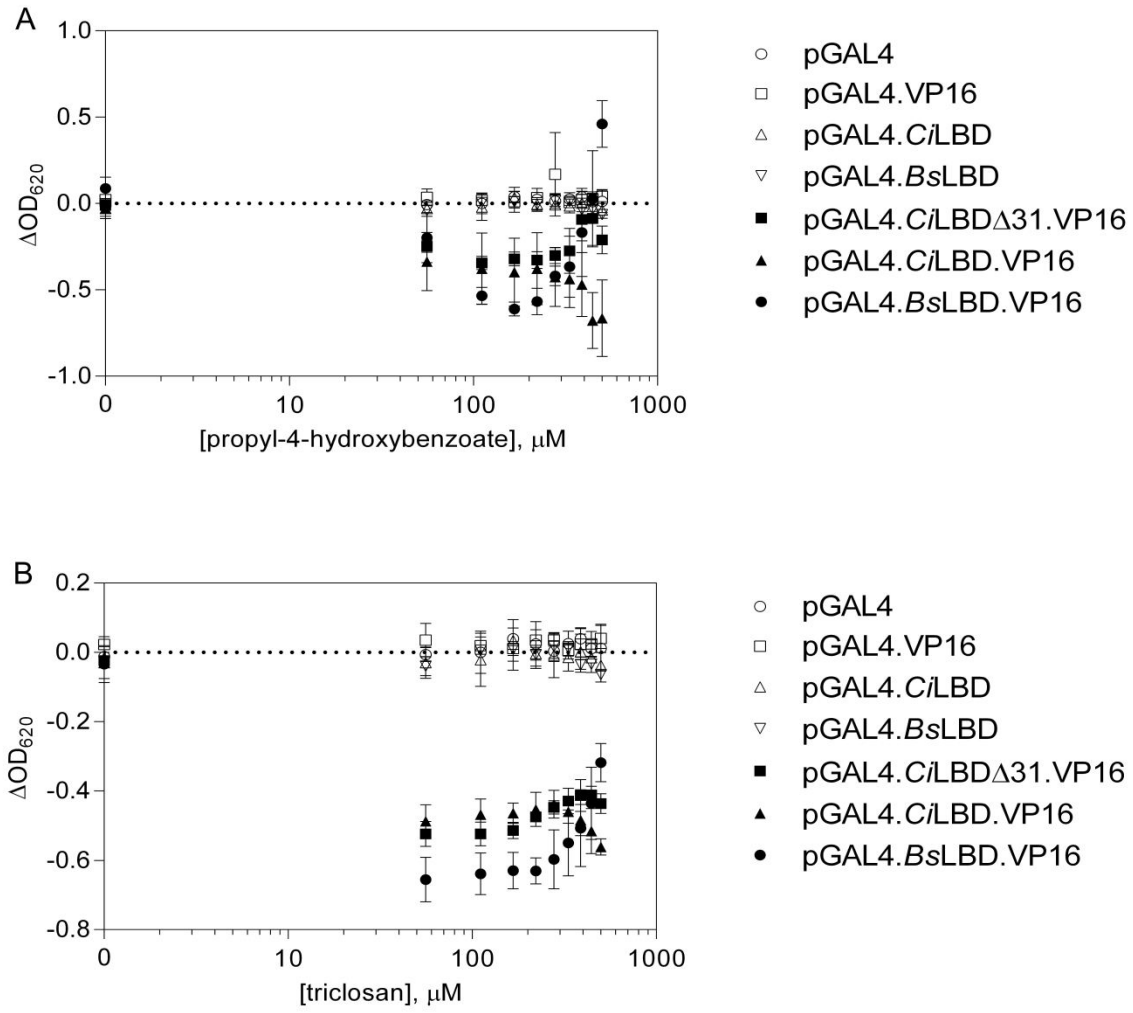
Abbreviations: DEET, *n*,*n*-diethyl-*m*-toluamide; DNC, did not compute; EDC, endocrine disrupting chemical; NI, no induction of β-galactosidase enzymatic activity; PAH, polycyclic aromatic hydrocarbon.

[a] Strains were incubated for 24 hours.

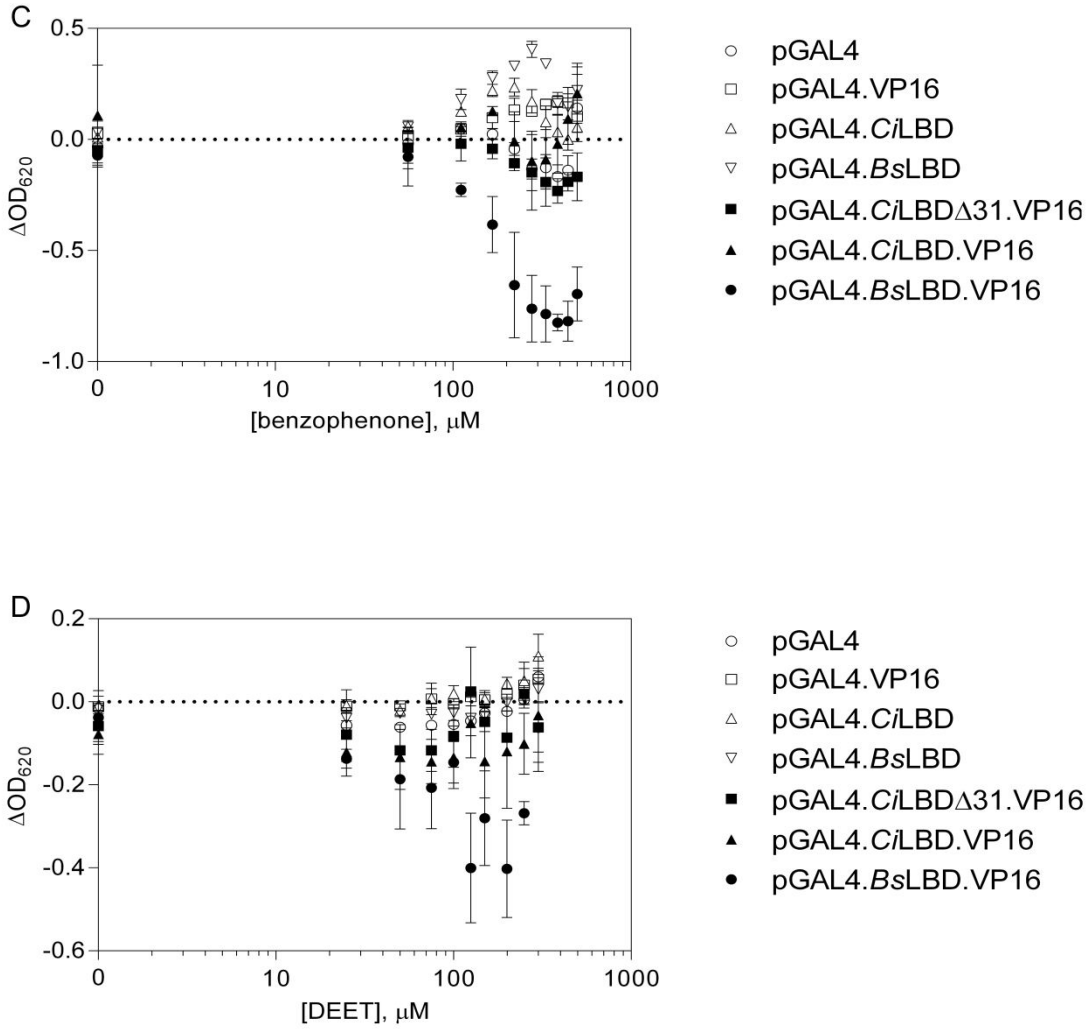
[b] Strains were incubated for 48 hours.

[c] EC_{50} and CI values for ketoconazole are given in nM.

APPENDIX SEVENTEEN



Appendix 17 Suppression of growth of recombinant yeast strains by: (**A**) propyl-4-hydroxybenzoate and (**B**) triclosan. Cell densities were quantified by OD_{620} values. Growth suppression (i.e. toxic) effects were detected by changes in OD_{620} values over time (ΔOD_{620} = OD_{620} after 48 h – OD_{620} after 0 h). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.



Appendix 17 (cont.) Suppression of growth of recombinant yeast strains by: (**C**) benzophenone and (**D**) *n*,*n*-diethyl-*m*-toluamide (DEET). Cell densities were quantified by OD_{620} values. Growth suppression (i.e. toxic) effects were detected by changes in OD_{620} values over time (ΔOD_{620} = OD_{620} after 48 h – OD_{620} after 0 h). Data points represent means of n = 3 replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

APPENDIX EIGHTEEN

Presentations at conferences and meetings:

Richter I & Fidler AE (2014) Tunicate xenobiotic receptors: application in high-throughput bioassays for bioactive compounds. Satellite Meeting Frontiers of Biology, Wellington, New Zealand.

Richter I & Fidler AE (2014) Tunicate xenobiotic-activated nuclear receptors: their application as sensor elements in high-throughput bioassays for marine microalgal biotoxins. International Conference on Harmful Algae, Wellington, New Zealand.

Richter I & Fidler AE (2014) Tunicate xenobiotic activated nuclear receptors: their application as sensor elements in high-throughput bioassays for marine microalgal biotoxins. New Zealand Marine Science Annual Conference, Nelson, New Zealand. Award for 'Best talk relevant to the Seafood Industry'.

Richter I & Fidler AE (2014) Sea squirts in bioassays— a tool to detect harmful marine biotoxins. A Conference for Women in Science, Wellington, New Zealand.

Richter I & Fidler AE (2013) Functionally significant polymorphisms in tunicate xenobiotic receptors. International Conference of Chemical Ecology, Melbourne, Australia.

Richter I & Fidler AE (2012) Expression and genetic diversity of tunicate xenobiotic receptors. Next-Generation Sequencing Conference, Dunedin, New Zealand.

Richter I & Fidler AE (2012) Expression and genetic diversity of tunicate xenobiotic receptors. Australian Centre for Evolutionary Biology and Biodiversity, University of Adelaide, Australia.

APPENDIX NINETEEN

Publications in refereed journals:

Richter I & Fidler AE (2014) Marine invertebrate xenobiotic-activated nuclear receptors: their application as sensor elements in high-throughput bioassays for marine bioactive compounds. Marine Drugs. 12(11): 5590-618. doi: 10.3390/md12115590.

Richter, I & Fidler AE (2015) Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast. Toxicon 95(0): 13-22. doi: 10.1016/j.toxicon.2014.12.011.

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Review

Marine Invertebrate Xenobiotic-Activated Nuclear Receptors: Their Application as Sensor Elements in High-Throughput Bioassays for Marine Bioactive Compounds

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Abstract: Developing high-throughput assays to screen marine extracts for bioactive compounds presents both conceptual and technical challenges. One major challenge is to develop assays that have well-grounded ecological and evolutionary rationales. In this review we propose that a specific group of ligand-activated transcription factors are particularly well-suited to act as sensors in such bioassays. More specifically, xenobiotic-activated nuclear receptors (XANRs) regulate transcription of genes involved in xenobiotic detoxification. XANR ligand-binding domains (LBDs) may adaptively evolve to bind those bioactive, and potentially toxic, compounds to which organisms are normally exposed to through their specific diets. A brief overview of the function and taxonomic distribution of both vertebrate and invertebrate XANRs is first provided. Proof-of-concept experiments are then described which confirm that a filter-feeding marine invertebrate XANR LBD is activated by marine bioactive compounds. We speculate that increasing access to marine invertebrate genome sequence data, in combination with the expression of functional recombinant marine invertebrate XANR LBDs, will facilitate the generation of

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Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast

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ABSTRACT

Marine microalgae can produce biotoxins that cause widespread poisoning in marine ecosystems and may also affect human health. While established microalgal biotoxins are detectable using chemical methods, a need remains for robust, inexpensive bioassays. Ligand-binding domains (LBDs) from a tunicate nuclear receptor, VDR/PXRα, which is orthologous to both the vertebrate pregnane X receptor (PXR) and the vitamin D receptor (VDR), can be activated by microalgal biotoxins when expressed in mammalian cell lines. Building on this observation, we developed a generic recombinant yeast bioassay platform that expresses chimeric proteins containing tunicate VDR/PXRα LBDs which mediate ligand-dependent transcription of a reporter gene (*lacZ*) encoding an easily assayed enzyme (β-galactosidase). Recombinant yeast strains expressing VDR/PXRα LBDs from two tunicate species, *Ciona intestinalis* and *Botryllus schlosseri*, were exposed to both synthetic and natural toxins. Structurally simple synthetic chemicals (*n*-butyl-*p*-aminobenzoate, carbamazepine, *p*-aminobenzoic acid, and bisphenol-A) generated EC_{50} values in the μM range, while more structurally complex marine biotoxins (okadaic acid, pectenotoxin-11, and portimine) activated the assays in the nM range. Given the large number of tunicate species, we propose that tunicate VDR/PXR LBDs may be used as 'sensor elements' in similar yeast-based high-throughput bioassays for detection of established microalgal biotoxins and uncharacterised marine bioactive compounds.

1. Introduction

Some marine microalgal taxa can produce highly toxic chemicals that, on occasion, cause widespread poisoning within marine ecosystems and which may also affect human health, particularly when accumulated in filter-feeding shellfish consumed by humans (MacKenzie et al., 2011; Wang, 2008; Wear and Gardner, 2001; Zhang et al., 2013). Over the past two decades internationally accepted methodologies for the detection of such natural marine biotoxins have become increasingly based on chemical, rather than biological, assays (Gerssen et al., 2010; McNabb et al., 2012; Suzuki and Quilliam, 2011). While such chemistry-based detection methods are generally reliable and highly specific, they require specialised equipment and skills along with detailed structural knowledge regarding the targeted biotoxins, and so do not allow detection of unknown marine biotoxins (Humpage et al., 2010; Nicolas et al., 2014). Although a range of *in vitro* bioassays for microalgal biotoxins have been developed (Banerjee et al., 2013; Bovee et al., 2011; Nicolas et al., 2014), currently the most widely-used microalgal biotoxin bioassay remains the unreliable and ethically questionable mouse mortality bioassay (Botana et al., 2009; Buckland, 2010; Stewart and McLeod, 2014). Consequently,

Abbreviations: AD, activation domain; AF-2, transcription activation function domain; BsVDR/PXRα, *Botryllus schlosseri* VDR/PXR orthologue α; BPA, bisphenol-A; CI, 95% confidence interval; CiVDR/PXRα, *Ciona intestinalis* VDR/PXR orthologue α; CID, PubChem compound accession identifier; CPRG, chlorophenol red-β-D-galactopyranoside; DBD, DNA-binding domain; DSP, diarrhetic shellfish poisoning; GAL4, yeast DNA-binding transcription factor; GM, growth media; LBD, ligand-binding domain; MCS, multiple cloning site; MM, minimal media; NR, nuclear receptor; NR1I, nuclear receptor subfamily 1 class I; PTX-2, pectenotoxin-2; PTX-11, pectenotoxin-11; PXR, pregnane X receptor; SAM, seeded assay media; VDR, vitamin D receptor; VP16, viral protein 16; XR, xenobiotic receptor.

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