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Review

The gruesome dance of contaminants and toxicity in organisms: the emerging science of chronoecotoxicology or wave ecotoxicology

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ABSTRACT

Life evolved to take advantage of external environmental cues, especially those of a recurrent cyclical nature, in order to assimilate energy and nutrients for survival. Given the continuous light-dark cycles owing to the Earth's rotation (circadian cycles), most life forms have adapted by synchronizing many of their physiological systems. These functions are governed and regulated at the biochemical, gene expression and post-transcriptional levels, which show wave-like behaviours. This review examines the role of biochemical oscillators in maintaining life processes and demonstrates how contaminants can influence these oscillations in the context of ecotoxicology. Circadian rhythms are one of the most important oscillatory mechanisms in organisms for optimal energy assimilation and metabolism. Circadian rhythms are synchronized with redox oscillators, such as peroxiredoxins (Prxs) and NADH/ATP, which regulate oxygen consumption (respiration), xenobiotic biotransformation and energy metabolism. Circadian rhythms are governed by clock genes (transcription factors) which in turn regulate many physiological functions that can determine the toxic outcomes of many contaminants. The toxicity of contaminants could be dramatically affected by the time of day of exposure to a given compound. Other oscillators show that cell activity could sustain oscillations at different frequency domains, and their potential to be disrupted by contaminants is explored. A case study is presented on the disruption of circadian rhythms in metallothioneins and peroxidases by cadmium exposure in freshwater mussels. Current evidence reveals that contaminants could disrupt the periodicity and intensity of rhythms in organisms. In conclusion, contaminants could influence the normal oscillatory pattern of biochemical functions and detoxification mechanisms in cells. Wave ecotoxicology or chronoecotoxicology consists in the study of the effects of xenobiotics on the cyclic or wave behaviour of biochemical systems of organisms and provides a more comprehensive view on the toxicity of xenobiotics.

KEYWORDS: oscillators, circadian rhythms, non-linear responses, ecotoxicity.

1. Introduction

Biochemical processes are often governed by non-linear changes in cells over time. Many of the molecular changes that underlie important physiological functions follow a clockwise (circadian) behaviour over the 24 hours of the day. Endogenous biological clocks are regulated by a negative feedback loop, as with biochemical oscillators, between clock genes and their corresponding proteins or metabolites [1]. Clock genes involve mostly transcription factors that govern cell metabolism, growth and division. The principal clock genes are listed in Table 1.

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Table 1. Genes involved in circadian rhythms.

Gene	Name	Role
Circadian Locomotor Output Cycles Kaput	CLOCK	Is a gene encoding a transcription factor (CLOCK) that is believed to affect both the persistence and period of circadian rhythms.
Aryl hydrocarbon receptor nuclear translocator	ARNT or BMAL1	The BMAL1 protein plays a key role as one of the positive elements in the mammalian autoregulatory transcription translation negative feedback loop (TTFL), which is responsible for generating molecular circadian rhythms involved in the regulation of energy metabolism (carbohydrates, lipids).
Period circadian protein	PER	The PER1 protein is important to the maintenance of circadian rhythms in cells, and may also play a role in the development of cancer. This gene is a member of the period family of genes. It is expressed with a daily oscillating rhythm, or an oscillation that cycles with a period of approximately 24 hours. PER1 is most notably expressed in the region of the brain called the suprachiasmatic nucleus (SCN), which is the primary circadian pacemaker in the mammalian brain.
Cryptochrome circadian regulator	CRY	This gene encodes a flavin adenine dinucleotide-binding protein that is a key component of the circadian core oscillator complex, which regulates the circadian clock. This gene is upregulated by CLOCK/ARNT1 heterodimers but then this upregulation is repressed in a feedback loop using PER/CRY dimers.
Nuclear receptor subfamily 1	nr1d1	This gene encodes a transcription factor of the nuclear receptor subfamily 1. The encoded protein is a ligand-sensitive transcription factor that negatively regulates the expression of core clock proteins.

In vertebrates, the transcription factors CLOCK and BMAL1 activate the expression of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. When the level of expression of PER and CRY proteins reaches a particular threshold, they translocate into the nucleus and inhibit the transcriptional activity of CLOCK-BMAL1 heterodimer, thereby blocking their own transcription. These internal molecular clocks are modulated by various environmental cues, such as daylight (or absence thereof), feeding/fasting, locomotor activity, sleep/wake and temperature. Light is a particularly important environmental cue as it permits organisms to adjust their feeding activity, predator avoidance behaviour and interaction with their community. Most organisms have evolved under this predictable light/dark cycle caused by Earth's rotation. The advantage of anticipating this cycle has driven the formation and evolution of endogenous molecular time-keeping mechanisms in response to this external cue. It has been shown that oxidation-reduction cycles of peroxiredoxin proteins constitute a universal marker for circadian rhythms in all life forms [2]. This process co-evolved with internal

control of redox homeostatic mechanisms, which followed the Great Oxidation Event some 2.5 billion years ago. Moreover, when organisms are isolated from external stimuli, they still exhibit self-sustained cycles in behaviour, physiology and metabolism with a period of about 24 hours, which suggests that biochemical processes are regulated by internal biological clocks.

The endogenous clock mechanisms that underlie circadian organization are conserved throughout phylogeny, from cellular organisms to plants, invertebrates and mammals. Thus, the integration of biological clocks into the cell's physiology was an important evolutionary advantage in organisms, since it allowed them to anticipate and adapt to cyclical changes in light, temperature and food availability. The resonance of internal molecular clocks or oscillators with external cues represents an important mechanism by which organisms adapt to their environment and could form the basis of toxicity with respect to endogenous time-keeping mechanisms and exposure concentration. In vertebrates, the time-keeping system comprises a series of biological clocks organized in a hierarchical manner. The main clock, or the so-called "master pacemaker", resides in the hypothalamus at the suprachiasmic nuclei (SCN), which process light signals from the exterior environment. The SCN then synchronize the other peripheral clocks in the body by the release of hormones or neuropeptides. These peripheral clocks are able to produce sustained circadian oscillations in physiological processes to control tissue-specific functions [3]. The increase in *clock* and *bmal1* genes led to increased expression of the activating elements CLOCK and BMAL1, which heterodimerize leading to the transcription of the *period* (*per* 1, 2 and 3) and *crytochrome* (cry1 and 2) genes. PER and CRY complexes inhibit their own transcription by binding to the CLOCK:BMAL1 heterodimer, which consists of the negative feedback loop mechanisms of circadian rhythms. Interestingly, circadian rhythms such as the daily recycling of oxidized peroxiredoxins, a family of thiol peroxidases, in nuclei-free red blood cells, could be maintained in the absence of gene transcription [3, 4]. Indeed, the oxidized/reduced state of peroxiredoxins changes during the 24-h period in red blood cells *in vitro* in the absence of external light (in the dark). Oxidized peroxiredoxins are reduced by the action of two enzymes: thioredoxin and sulfiredoxin reductases, which require NADH and ATP as co-substrates, respectively. Their activities also follow a diurnal pattern of changes. It appears that between 2% and 10% of genes follow circadian rhythms [5]. It was found that hundreds of metabolites fluctuate during the day/night in a circadian manner [6]. The metabolites cover the entire spectrum of intermediates such as amino acids, carbohydrates, lipids and nucleotides. Many of them canonically follow the expression of clock-associated genes, suggesting a tight regulation between gene expression and the formation of corresponding metabolites.

In this respect, it is not surprising that the toxicological responses of organisms to various environmental contaminants could be influenced by circadian rhythms or other wave behaviour at the molecular/physiological level. This has given rise to the emerging fields of chronopharmacology, the study of the circadian modulation of the therapeutic effects of drugs, and chronotoxicology, the study of the sensitivity to toxic xenobiotics with respect to the circadian period. Wave ecotoxicology or chronoecotoxicology is the study of the impact of contaminants on the normal cyclic behaviour of biological functions in the environmental context and is the topic of this review. Indeed, not only could the sensitivity of organisms to contaminants change at a given point in a physiological cycle, but circadian rhythms could be directly disrupted by contaminants as (a less understood) pathway to health loss.

The response of organisms to stress is governed by glucocorticoids (cortisol) in higher organisms (vertebrates). During stressful situations where organisms have to adapt to unexpected events, cortisol is secreted in the blood stream, producing various effects, such as gluconeogenesis, to increase glucose levels, ion transport, water retention and immunosuppression [7]. Glucocorticoids could also increase the expression of genes involved in circadian rhythms (Per1 and nr1d1) and downregulate expression of the proteinaceous egg yolk precursor vitellogenin, which is under the control of the 17β-estradiol receptor [8]. This suggests that changes in stress

response and in circadian rhythms could modulate other pathways, such as vitellogenesis during gametogenesis in oviparous organisms. Feeding and fasting activities also follow a 24-h cycle. In a study looking on different feeding strategies in wedge sole, under a self-feeding regime, 91% of feeding demands occurred during the dark phase [9]. However, fish under the self-feeding regime consumed less food than fish under the diurnal feeding and nocturnal feeding regimes and had higher mortality. Plasma cortisol levels were dramatically increased in diurnal feeding and self-feeding regimes compared to the nocturnal feeding regime. Growth rates were optimal with the nocturnal feeding regime, suggesting that this species synchronized its feeding with dark periods. In a study in Senagalese sole, daily cycles in plasma cortisol, blood glucose and locomotor and self-feeding activities were examined [10]. Under normal light/day conditions, plasma cortisol oscillates, with the acrophase (i.e., maximum peak level) at 36 ng/mL at 15:00 h and the bathyphase (i.e., minimum levels) at 5 ng/mL during the night. This cortisol rhythm persisted under constant light conditions, with lower values for the acrophase at 7 ng/mL and shifted to 18:00 h. With respect to locomotor and self-feeding activity, a marked rhythm was observed for the light/dark treatment, with higher activity observed during the night, with acrophases located at 02:14 h and 02:37 h, respectively. This suggests that during low cortisol levels (unstressed conditions), feeding and locomotor activities are optimal. From an environmental perspective, exposure to corticosteroid drugs could pose a risk to fish at the circadian level. Adult zebrafish were exposed for 21 days to a commonly prescribed corticosteroid, fluodrocortisone acetate (0.006-42 µg/L) to determine whether exogenous exposure could have detrimental effects on fish physiology [11]. Although no remarkable impacts were observed on reproduction (ovary growth) or the hypothalamic-pituitary-gonadal-liver axis, plasma glucose level and blood leukocyte numbers were increased and decreased, respectively at concentrations as low as 42 ng/L fluodrocortisone acetate. Moreover, the brain circadian network was altered as evidenced by strong decreases in per1a and nr1d1 transcript levels at concentrations of 6 and 42 ng/L. Hatching success, heartbeat and swimming activity were all increased at concentrations >42 ng/L. These responses suggest that circadian signals could be disrupted by environmental exposure to cortisol-mimicking compounds. Indeed, glucocorticoids were shown to modulate the expression of genes involved in the circadian signaling systems and to alter the hepatic oscillator involved in energy metabolism and detoxification activities [12]. In fish maintained under a 12-h photoperiod, an *ip* injection of dexamethasone at Zeitgeber (i.e., time giver) time 2 induced per1 genes while it decreased bmal1a and clock1a gene expression in the liver at 8 h post-injection. Water salinity was also able to alter plasma cortisol levels in Senegalese sole (*Solea senegalensis*), but it was not determined if the circadian rhythm was affected [13]. Salinity changes occur in estuaries during tidal activity, which occur 2 to 3 times over a 24-h period, and the intensity of the tides also varies during the month (full moon), providing important external cyclical cues for estuarine fish.

The circadian clock requires tight coordination between the transcriptome and metabolome [6]. Given that food is a potent Zeitgeber for peripheral clocks, many nutritional metabolites are implicated as transducers of circadian time in the liver. From a dataset of over 500 metabolites, metabolites and intermediates within the carbohydrate, amino-acids/protein, lipid, nucleotide and xenobiotic pathways exhibited an oscillatory behaviour in a circadian manner. For example, the uracil salvage pathway followed the expression of clock genes. Amino acids and xenobiotic metabolism peaked after 15:00 h while nucleotides, carbohydrates and lipids peaked early in the morning at 09:00 h. Deficiency of circadian clock genes in mutant mice significantly reduced lifespan and increased degenerative (oxidative?) damage of eye lens leading to cataracts [14], thus, showing the importance of maintaining biological clocks in healthy organisms.

2. Circadian behaviour of redox homeostasis

The redox status in cells follows a daily rhythmic behaviour and is thought to have originated at the time of the Great Oxidation Event between 2.5 and 3 billion years ago [15]. This forced organisms to adjust aerobic metabolism and reactive oxygen

metabolism with light and day cycles from Earth's rotation. The steady rise of oxygen in the biosphere by photosynthetic bacteria (cyanobacteria) has forced organisms to develop means of handling harsh oxidizing conditions. This process is driven by the day-night cycle, which introduced a circadian dynamic in organisms exposed to oxygen and the implementation of redox homeostasis mechanisms co-evolved with a circadian period. Evidence of circadian oscillations in redox homeostasis was reported in the early days of the science of chronobiology [16]. Even before the identification of clock genes, studies in rodents revealed that reduced glutathione (GSH) and NADPH levels display diurnal cycles in their oxidation state (GSSG and $NADP^+$) [17]. Given that the production of reactive oxygen species (ROS) is a common pathway of toxicity leading to many pathophysiological conditions in organisms, it is sometimes considered a universal mode of action of xenobiotics [18, 19]. ROS production normally occurs in mitochondria during respiration and energy production, which require tight regulation of ROS levels in cells. Indeed, the survival of all aerobic life forms requires the ground state of molecular oxygen, O_2. However, many xenobiotics could enhance the normal production of ROS, which could lead to oxidative damage such as lipid peroxidation, protein inactivation and DNA damage. This is the O_2 paradox i.e., aerobic prokaryotic and eukaryotic organisms require O_2 to live, yet produce ROS in the process, which are deleterious in the long term. To survive in such an environment, organisms have developed an array of antioxidant strategies (ascorbate, vitamin E, glutathione, superoxide dismutase, catalase and peroxidases) to protect themselves against the harmful effects of ROS in a cell's environment. Oxidative stress has not only been implicated in the toxicity of many xenobiotics but is involved in a wide variety of degenerative conditions, diseases and syndromes, including mutagenesis, cell transformation, cancer, atherosclerosis, chronic inflammatory diseases and central nervous disorders (dementia and Parkinson's disease).

Peroxiredoxins (Prxs) are ubiquitous and abundant antioxidant enzymes with peroxidase activity involved in the elimination of H_2O_2 (ROS) production during metabolism, and they follow circadian cycles of oxidation-reduction. Prxs are a highly conserved family of antioxidant proteins that help to control intracellular levels of peroxide levels [20]. The peroxidase properties of Prx proteins is assured by the presence of cysteine residues at the catalytic active site and are oxidized to sulphenic acid (Cys-SOH) by ROS which is reduced back to Cys-H by thioredoxin. The oxidized thioredoxin is then recycled back to reduced thioredoxin by NADPH-dependent thioredoxin reductase. The cysteine moiety of Prx could be further oxidized by ROS to the sulphinic (Cys-SO2H) and sulphonic (Cys-SO3H) acids. Sulphinic could be reduced back to cysteines by ATP-dependent sulphiredoxin reductase, while the sulphonic acid form is considered irreversibly damaged. The oxidative (sulphenic and sulphinic acids) form of Prx has been shown to follow a circadian rhythm in cells with the reduced form of Prx [21]. The cycling of Prx redox status is coupled not only to canonical circadian gene expression pathways but also to a noncanonical transcript-independent circadian clock, suggesting that time-keeping mechanisms could be independent of gene regulation. Because the regulation of redox status in cells follows a 24 h cycle, it is not known whether compounds that produce oxidative stress could disrupt time-keeping mechanisms in cells.

Cadmium (Cd) is a well-known toxic divalent heavy metal that can lead to oxidative stress by producing ROS in a Fenton-like reaction [22]. Moreover, Cd is readily sequestered and eliminated by cysteine-rich metal binding proteins coined metallothioneins (MTs). In rats exposed to Cd interperitoneally at 8 different times of the day, tissue distribution and hepatic metallothionein (MT) levels differed when administered during dark hours [23]. Testis contained six times more Cd when administered in the dark, and MT levels were higher. This suggests that the organism's response to toxic metals like Cd could change during the day and that the Cd detoxification mechanism could perhaps be related to diurnal influences. Indeed, the diurnal variations in mRNA levels of MT-1 and MT-2 in the liver of mice changed up to 40-fold with respect to the lowest levels [24]. The diurnal variation of MT mRNAs closely followed the clock gene albumin D site-binding protein and was anti-phase to the

clock gene ARNT-like protein-1 (BMAL1) in liver and kidney. Hepatic protein content in MT was also cyclic and the acrophase corresponded to a 3-fold difference. This suggests that the time of exposure could influence the capacity of the body to defend against toxic chemicals. Given that MT is also involved in the sequestration of ROS [25] and that the redox status oscillates during the day, it is expected that MT levels could be modulated by circadian motion in redox mechanisms. Glucocorticoids were also shown to increase MT expression in mammals and fish, which is another mechanism by which MT could be influenced by circadian cycles and the stress response which involves cortisol release [26, 27].

The oxidative status is associated with lifespan, metabolism and circadian rhythms in aquatic organisms [28]. The antioxidative enzyme activities (peroxidases, superoxide dismutase and catalase) and lipid peroxidation (LPO) in the liver of trout and sturgeon changed though the day, with increased hepatic LPO levels during the dark hours for sturgeon, which has a higher life expectancy than trout. However, the higher activity (and efficiency) of the antioxidant defences in the brain of sturgeon resulted in less LPO than in trout. Moreover, the absence of daily rhythms in plasma cortisol levels in sturgeon could indicate lower susceptibility to stress and suggests that the mechanisms involved in cortisol release could differ between species (chrondrosteans vs telosteans). The cyclic changes in ROS levels in organisms could also influence detoxification response in salmon [29]. Gene expression of catalase and glutathione peroxidase oscillates during the day, showing highest expression levels for the first 2 h of the day (light) and lowest expression levels between 10:00 h and 14:00 h in the day. Interestingly, gene expression in heat shock protein 70 paralleled the observed changes in the above antioxidant gene expression. In salmon exposed to a high concentration of H_2O_2 (1500 mg/L) for 20 min to treat skin infections at different times of the day, different physiological responses were observed. Glucose, lactate and cortisol levels were higher in treated fish in daytime compared to the corresponding controls. Gene expression of antioxidant enzymes (glutathione peroxidase, catalase and Mn-dependent superoxide dismutase) in the liver displayed daily cyclic changes in both treated and control groups and showed higher mRNA expression levels in salmon treated with H_2O_2 at Zeitgeber time of 6 h (i.e., 6 h after light exposure). In gills, rhythmic expression was only found for glutathione peroxidase in the control fish and for heat shock protein 70 and for Mn-dependant superoxide dismutase in the H_2O_2-treated groups. The acute stress response to air exposure in gilthead sea bream is time-of-day dependent [30]. Since most physiological functions show circadian rhythms, it is not surprising that environmental stressors might have different effects depending on the timing of exposure. In fish submitted to air exposure for 30 s and then returned to water for 1 h before samples were taken (sampled every 4 h during a 24-h cycle), a daily rhythm in plasma cortisol was observed, with the acrophase during the dark hours. Interestingly, a daily rhythm in plasma cortisol was not observed in the control fish. Conversely, blood glucose showed daily oscillations in the control group but not in the air-exposed treatment group, but showed higher values at all sampling times. In the hypothalamus of control fish, a daily rhythm in corticotropin-releasing hormone gene expression was observed, with the acrophase at the beginning of the light phase. However, this rhythm was lost in stressed fish. These studies reveal that toxicity depends on the time of day the stressor is applied and that the cyclic redox status could influence the toxic outcomes of environmental stressors. This suggests that xenobiotic detoxification pathways could be influenced by circadian rhythms as well.

3. Circadian control of xenobiotic biotransformation

In addition to the circadian influence of MT expression which is involved in metal detoxification, its expression could be changed by inflammation and cortisol levels which are also circadian. Recent evidence suggests that xenobiotic detoxification pathways are also influenced by circadian cycles. In hepatocellular carcinoma (HCC), both MT and circadian genes are dysregulated, which suggest they play a role in proliferative disease formation [31]. Indeed, downregulation of MT-1, MT-2 and metal

transcription factors were observed in hepatocellular carcinoma compared to normal livers. MTs are considered a biomarker HCC and have typical circadian rhythms. HCC produced a dramatic decrease in the expression of clock genes such as CLOCK and BMAL1 and decreased the expression of the clock feedback control genes PER1, PER2 , CRY1 and CRY2. However, Nr1d1 and D-box-binding protein were upregulated in liver carcinomas compared to normal livers.

Melatonin is an important regulator of endocrine and circadian rhythms in vertebrates [32, 33]. Melatonin is produced from the oxidation of serotonin, which is produced from the amino-acid tryptophan and follows circadian cycles in fish. The influence of the classic aryl hydrocarbon receptor (AhR) agonist 2,3,7,8-tetracolorodibenzo-p-dioxin (TCDD) on melatonin metabolism in fish hepatocytes was examined in rainbow trout liver cells [34]. Exposure of fish hepatocytes to this AhR agonist increased the amount of the hydroxylated metabolite of melatonin (6-hydroxymelatonin) by about 2.5-fold after 24 h. This suggests that exposure to AhR agonists could alter circadian melatonin signaling in fish liver. The non-steroidal anti-inflammatory drug diclofenac is commonly found in wastewater effluents and has been shown to activate AhR, which induces transcription of cyp1A cytochrome [35]. The AhR and hypoxia inducible factor 1 were shown to interact with proteins involved in circadian rhythms in fish. Three-spined sticklebacks were exposed to 1 µg/L diclofenac for 14 days and samples were taken at three time points during the light period in order to find disturbances in the daily variation in metabolism. Diclofenac induced cytochrome P4501A activity and altered the mRNA levels in circadian genes in the following manner: at 11 h of daylight, PER1 and CLOCK mRNA levels were higher in diclofenac-treated fish.

The proline-rich basic leucine zipper (PARbZip) transcription factors (albumin D site-binding protein, hepatocyte leukemia factor, thyrotroph embryonic factor) which bind to DNA elements of the consensus sequence 5'-RTTAYGTAAY-3' (where R is G or A and Y is C or T) as homo or heterodimers, are expressed in a highly circadian manner [36, 37]. PARbZip transcription factors regulate the expression of many target genes involved in xenobiotic detoxification in central and peripheral tissues (Table 2). In addition to those listed in Table 2, other related enzymes such as d-aminolevulinate synthase 1 (ALAS1) and cytochrome P450 oxidoreductase (POR), two enzymes required for the activity of all forms of class I monooxygenases, were also influenced by circadian cycle. Moreover, PARbZip proteins drive the circadian expression of peroxisome proliferator activated receptor α (PPARα) and constitutive androgen receptor (CAR), which are also involved in drug metabolism. The circadian expression of the constitutive androgen receptor manifests itself in a strongly daytime-dependent manner. The induction of Cyp2B10 mRNA by pentobarbital in the liver of mammals was attenuated in PARbZip knockout mice [36]. PARbZip proteins are mainly regulated by BMAL1, CLOCK, CRY1,2 and PER1,2 [38]. In fish acclimated to 24-h dark conditions, the expression of phase I, II and III biotransformation enzymes were influenced by light and dark cycles, although dark-acclimated fish showed a different circadian cycle, which suggests the co-existence of light-independent biological clocks in zebrafish liver; perhaps the circadian cycle of redox status in cells [37]. For phase 1 biotransformation, glutathione S-transferase 1 (gstr1) gene expression acrophased at the first 2 h of light, while in dark-acclimated fish the acrophase was between 18 and 22 h in the dark. For the phase II enzymes, sulfotransferase 2 (sult2) gene expression acrophased at the first 2 h of light with a second peak at the first 2 h of dark in fish acclimated to the normal light/day cycle while, in dark-acclimated fish, only one peak in its expression was observed between 18:00 h and 22:00 h. In the case of smtB gene expression which encodes for MT-II, the acrophase also occurred within the first 2 h of light exposure, with a second minor peak at 10 h. In fish acclimated in the dark, the second peak occurred 8 h later, suggesting that circadian rhythms are influenced by light and dark cycles, but still remain in the absence of light and dark cycles. The master clock in the brain controls or synchronizes the clock of the liver, which is the major organ for xenobiotic biotransformation [39]. In mutant mice lacking circadian clock activity and challenged with acetaminophen

Table 2. Detoxification enzymes and transporters under circadian control.

Detoxification genes	Function/role
Phase 1	
CYP family	Represented by over 40 types of cytochrome P450s in mammals but the number differs between taxa (annelids, bivalves, crustacean and fish). Major enzyme complex responsible for monooxygenase reactions i.e., involved in the addition of one oxygen to xenobiotics. CYP1A and CYP3A enzymes are noteworthy for the hydroxylation of coplanar aromatic hydrocarbons and heterocyclic aliphatic hydrocarbons, respectively.
Aminolevulinic acid synthase	This enzyme (EC 2.3.1.37) is involved in the catalysis production of D-aminolevulinic acid, the precursor for the biosynthesis of tetrapyrroles such as hemes, cobalamins and cytochromes. Its activity is required for the concerted production of hemoproteins such as peroxidases and cytochromes P450.
NADPH cytochrome P450 reductase	A membrane-bound enzyme in the endoplasmic reticulum required for electron transfer from NADPH to cytochrome P450 of the eukaryotic cell. Since all microsomal P450 enzymes require this enzyme for activity, it is expected that disruption of its expression could have devastating consequences towards xenobiotic metabolism.
Aminopyrine N-demethylase	A cytochrome P450 involved in the demethylation of aminopyrine in mammals and fish. Aminopyrine is a pyrazolone analgesic, anti-inflammatory, and antipyretic agent and has been used as a non-invasive measure of cytochrome P-450 metabolic activity in liver function tests.
NAPDH quinine oxidoreductase/menadione reductase	Quinine 3-monooxygenase (EC 1.14.13.67) is an enzyme that catalyzes the oxidation of quinine by NADPH and O_2 giving 3-hydroxyquinine. This enzyme is also called quinine 3-hydroxylase. Quinine is a natural alkaloid with antipyretic, analgesic activities and used to control malaria. This enzyme complex could use other substrates such as menadione (NADPH menadione oxidoreductase)
Epoxide hydrolases	They are also known as epoxide hydratases, and are enzymes that metabolize compounds that contain an epoxide residue; they convert this residue to two hydroxyl residues through a dihydroxylation reaction to form diol products. The hydrolases are distinguished from each other by their substrate preferences and their physiological functions.
Phase II	
Sulfotransferases	From the transferase enzymes family involved in the transfer of a sulfate group from a donor molecule to an acceptor alcohol or amine. The most common physiological sulfate donor is 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Steroids containing hydroxyl group are often targeted by this enzyme which lead to inactivation.
UDP-glucuronyltransferase	Is a cytosolic glycosyltransferase (EC 2.4.1.17) that catalyzes the transfer of the glucuronic acid component of UDP-glucuronic acid to a small hydrophobic molecule. This glucuronidation reaction is arguably the most important of the Phase II (conjugative) enzymes.
Glutathione S-transferases	This enzyme is responsible for the primary line of defence against both acute and chronic toxicities of electrophiles and reactive oxygen/nitrogen species. GSTs are a superfamily of Phase 2 detoxification enzymes that detoxify both ROS and toxic xenobiotics, primarily by catalyzing GSH-dependent conjugation and redox reactions

Table 2 continued..

Detoxification genes	Function/role
Phase II	
N-acetyltransferase	N-acetyltransferase is an enzyme that catalyzes the transfer of acetyl groups from acetyl-CoA to arylamines. They have wide specificity for aromatic amines, particularly serotonin, and can also catalyze acetyl transfer between arylamines without CoA. The transfer of acetyl groups is important in the conjugation of metabolites from the liver, to allow excretion of the by-products (phase II metabolism). This is especially important in the metabolism and excretion of drug products.
Phase III	
P-glycoprotein	P-glycoprotein 1 (permeability glycoprotein, abbreviated as P-gp or Pgp) also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1). They are important proteins of the cell membrane that pumps many foreign substances out of cells (efflux). More formally, it is an ATP-dependent efflux pump with broad substrate specificity. It exists in animals, fungi and bacteria and likely evolved as a defense mechanism against harmful substances.
Organic anionic transporter	The organic anion transporter 1 also known as solute carrier family 22 member 6 (SLC22A6) is a protein involved in the efflux of anionic compounds from cells. It is a transmembrane protein that plays a central role in organic anion transport in kidneys but in other organs as well. Homologs for this transporter have been identified in rats, mice, rabbits, pigs, fish, and nematodes.
Receptor expression	All these receptors have the modular structure of steroid receptors and most of them bind to their cognate DNA elements as heterodimers of the retinoid X receptor.
Aryl hydrocarbon receptor (AhR)	The AhR is a ligand-activated transcription factor involved in the regulation of biological responses to planar aromatic (aryl) hydrocarbons. This receptor has been shown to regulate xenobiotic-metabolizing enzymes such as cytochrome P450. AhR binds to several exogenous ligands such as natural plant flavonoids, polyphenolics and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds. AhR activity could lead to bioactivation in which the induction of metabolizing enzymes results in the production of toxic metabolites (benzo(a)pyrene).
Metal binding transcription factor element (MTF)	From insects to mammals, metallothionein (MT) genes are induced in response to heavy metal load by the transcription factor MTF, which binds to short DNA sequence motifs, termed metal response elements. It plays a direct role in heavy metal detoxification and acquisition by inducing the expression of MT and transport of metals.
Constitutive androstane receptor (CAR)	CAR is a member of the nuclear receptor superfamily and along with pregnane X receptor (PXR) functions as a sensor of endogenous and xenobiotic substances. In response to androgen-like compounds, the expression of proteins responsible for the metabolism and excretion of these substances is upregulated. Hence, CAR and PXR play a major role in the detoxification of foreign substances such as drugs and androgenic steroids (testosterone). Androstenol and several isomers of androstanol, androstanes, are endogenous antagonists of the CAR.

Table 2 continued..

Detoxification genes	Function/role
Receptor expression	
Pregnane X receptor (PXR)	PXR is a nuclear receptor involved in "sensing" the presence of foreign toxic substances and in response up-regulate the expression of proteins involved in the detoxification and clearance of these substances from the body. PXR belongs to the nuclear receptor superfamily, members of which are transcription factors characterized by a ligand-binding domain and a DNA-binding domain. PXR is a transcriptional regulator of the cytochrome P450 gene CYP3A4, binding to the response element of the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor RXR. It is activated by a range of compounds that induce CYP3A4, including dexamethasone and rifampicin.
Peroxisome proliferator activated receptor (PPARs)	The PPARs are a group of nuclear receptors regulating the expression of genes involved in the regulation of cellular differentiation, development, and metabolism (carbohydrate, lipid, protein) and tumorigenesis. PPARs heterodimerize with the retinoid X receptor (RXR) and bind to specific regions on the DNA of target genes. These DNA sequences are termed PPREs (peroxisome proliferator hormone response elements). The retinoid X receptor also forms a heterodimer with a number of other receptors (e.g., vitamin D and thyroid hormone).
Liver X receptor	This receptor is a member of the nuclear receptor family of transcription factors and is closely related to nuclear receptors such as the PPARs and retinoic X receptor. Liver X receptors (LXRs) are important regulators of cholesterol, fatty acid, and glucose homeostasis. LXRs bind to oxysterols as ligands.
Farnesoid X receptor	This receptor is expressed at high levels in the liver and intestine. Deoxycholic acid and other bile acids are natural ligands for the receptor. Similar to other nuclear receptors, when activated, FXR translocates to the cell nucleus, forms a dimer (in this case a heterodimer with RXR) and binds to hormone response elements on DNA, which up- or down-regulate the expression of genes, such as cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis from cholesterol. They have been involved in the regulation of hepatic triglyceride levels.
Hepatocyte nuclear factor	This protein factor regulates the expression of a wide variety of target genes and functions in the liver, such as development and metabolic homeostasis of the organism. It can also influence expression of the insulin gene as well as genes involved in glucose transport and metabolism.
Heat shock proteins (HSP)	HSP are a family of proteins expressed in response to exposure to stressful conditions such as heat/cold, UV light and chemicals. Many members of this group perform chaperone functions by stabilizing new proteins to ensure correct folding or by helping to refold proteins that were damaged by cell stress. The dramatic increase of the heat shock proteins is a key part of the heat shock response and is induced primarily by heat shock factors which are found in all living organisms.

(hepatoxicant) and phenobarbital (metabolized by cytochrome P4502A), CLOCK-deficient mice were remarkably resistant to acetaminophen toxicity and displayed longer phenobarbital-induced sleep time, while PERIOD-deficient mice had a short phenobarbital-induced sleep time. In hepatocytes from mice mutant with a null allele of brain and muscle clock regulation (Bmal1, Arntl), the toxicity of acetaminophen was dramatically reduced owing to reduced bioactivation from

decreased NADPH-cytochrome P450 oxidoreductase gene expression and activity [40].

These results suggest that the periodic nature of xenobiotic biotransformation could likely modulate the toxicity of some compounds. Indeed, the toxicity of cadmium in mice was shown to follow a diurnal variation as with MT levels [41]. Mice maintained under normal conditions (12 hr light/ dark cycle, lights on at 08:00 h), were injected intraperitoneally with a toxic dose of $CdCl_2$ (7.2 mg/kg) at different times during the day. Interestingly, mice injected at 08:00 h were sensitive to $CdCl_2$ injection after 14 days while mice injected at 16:00 h, 20:00 h and midnight were more tolerant. Although MT levels were not significantly affected, a similar pattern with hepatic GSH levels was observed, which were low in sensitive mice and higher in tolerant mice. In a subsequent study, when mice were injected with 6.4 mg/kg of $CdCl_2$ at 02.00 h all died but when injected at the same dose at 18:00 h, they all survived [42]. Again, hepatic GSH was higher in resistant mice, and difference in hepatic Cd level, basal MT levels and speed of induction of MT were not involved in Cd-resistance. It is possible that diurnal change in redox status (peroxiredoxins which require reduced GSH) could modulate the impacts of Cd-induced production of ROS.

4. Other ecotoxicologically-relevant functions

We found other toxicologically-relevant physiological systems to be under circadian control. The immune defences in fish were found to have a circadian behaviour which could modulate the fish susceptibility to disease [43]. Fish (*Oreochromis niloticus*) were acclimated to 2 photoperiod conditions for 20 days: 12L:12D (LD) and constant dark during 24 h (DD). Plasma lysozyme and peroxidase activities exhibited rhythmicity in fish under LD but not in fish under DD. In fish injected with a bacteria endotoxin lipopolysaccharide (LPS) at Zeitgeber time 3 (ZT3; day) and at Zeitgeber time 15 (night), the magnitude in the increase in both lysozyme and peroxidase activities was stronger at ZT3. Plasma cortisol levels were higher only in fish exposed to LPS at ZT3 but melatonin content was only increased in LPS-injected fish at ZT15. These studies suggest that photoperiod could desynchronize the expression of humoral factors and stress responses in fish. In a previous study with *Oreochromis niloticus*, serum peroxidases and lysozyme were increased between 02:00 h and 06:00 h of the day (light) and serum cortisol exhibited elevated levels only between 22:00 h and 06:00 h, once again highlighting the rhythmic functioning of the immune system in fish [44]. The production of the sex steroid hormones testosterone and 17β-estradiol was also shown to oscillate during the day [45]. During the breeding season of the sex-changing bluebanded goby, plasma cortisol and sex steroid hormone levels changed during the day. Cortisol concentrations were lowest at the 08:00-10:00 h sampling point and acrophased in late morning (10:00-12:00 h). Levels of 17β-estradiol were elevated at the 18:00-20:00 h sampling point, but testosterone and 11-ketotestosterone levels were elevated in the morning. In the context that organisms could be exposed to estrogenic compounds from municipal effluents [46] during feeding activity early in the morning, the estrogenic effects could dampen the testosterone effects during these hours and contribute to intersexuality where males and females are not completely formed. Fluctuations in metabolic and neuroendocrine mediators could also influence the circadian levels of urea excretion in the mangrove killifish *Rivulus marmoratus* [47]. Urea excretion was highest between 12:00 h and 18:00 h and was reduced by exposure to serotonin receptor and cortisol receptor antagonists, suggesting that serotonin and cortisol signaling were required for urea excretion. Repeated exposures to these antagonists did not abolish the circadian rhythm, but severely decreased the amplitude of changes. Hormones found in the environment also have the potential to disrupt neural networks involved in circadian rhythms. Progestins and corticosteroids significantly decreased locomotor activity in *Danio rerio* embryos, whereas estradiol-17β increased locomotor activity. The expression of clock genes (per1a and nr1d2a) was induced by progestins and cortisol, while estradiol-17β produced the opposite effect. These studies show not only that the toxicity of chemicals could be altered depending on the time of day exposure is initiated but also that chemicals could alter circadian rhythms as well.

5. Xenobiotic-induced alterations in circadian rhythms

This section focuses more directly on the effects of xenobiotics on biological clocks and oscillators rather than on the reverse, i.e., the influence of biological clocks in the manifestation of toxicity. In rats acclimated to 12-h light and 12-h dark cycle, Bmal1 and Clock expression peaked at early scotophase (dark period of light/dark cycle), Per1 peaked at the end of the day and Per2 and Cry2 expression peaked at mid-scotophase [48]. When exposed to Cd, the pattern was significantly disrupted for Clock and Bmal1 or changed in phase for Per1, Per2 and Cry2. Interestingly, melatonin (a sleep-inducing compound) reversed the effects of Cd for Per1 expression only. Cd also disrupted the 24-h cycle of Cu/Zn-superoxide dismutase, catalase, nitric oxide synthase, heme oxygenase1 and 2, with melatonin addition counteracting these effects. In the case of catalase and heme oxygenase 2, Cd exposure increased their expression while the addition of melatonin returned gene expression to the levels of control rat. This suggests that Cd could influence the circadian redox status in cells. Cd was also shown to suppress 24-h rhythmicity in expression of the adenohypophysial prolactin gene and in circulating prolactin at the start of day only [49]. Cd treatment effectively disrupted the 24-h variation in the expression of every pituitary parameter (prolactin, luteinizing hormone, thyrotropin and corticosterone) except for metallotheinein-3. Cd also suppressed daily rhythm of pituitary Bmal1, Per1, Per2, Cry1 and Cry2. Co-administration of melatonin restored rhythmicity in Clock and Bmal1 expression but shifted the acrophase in pituitary Per1, Cry1 and Cry2 gene expression to the scotophase. Melatonin also counteracted the effects of Cd on the circadian levels of plasma prolactin, corticosterone, thyrotropin and luteinizing hormones.

Exposure to endocrine disrupting compounds was shown to disrupt lipid metabolism and circadian rhythms in zebra fish [50]. Exposure to tributyltin, 2 commercial flame retardants and a UV-filter agent found in sunscreens altered both the circadian clocks and lipid metabolism in developing zebrafish, which suggests that some obesogenic chemicals could also disrupt biological rhythms in fish given that lipid accumulation coincided with loss of periodicity in clock genes. This is consistent with the accumulation of lipids in the liver of fish exposed to a primary-treated municipal effluent which contains many of these pollutants [51]. The effects of released natural and synthetic steroid hormones on the core clock genes were examined in zebrafish embryos [52]. Progestins and cortisol decreased the intensity of expression of core clock genes while 17β-estradiol had the opposite effect. Chronic copper exposure leads to the loss of circadian rhythm during dietary exposure that involves the lack of response to circulating melatonin and a loss of circulating serotonin in addition to chronic physiological stress such as cortisol liberation in the plasma [53]. The metabolism of melatonin was shown to be altered in fish hepatocytes exposed to the AHR-agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [34]. Treatment of hepatocytes with TCDD resulted in the increased levels of 6-hydroxymelatonin about 2.5-fold after 24 h when compared to control cultures suggesting that AhR agonists could disrupt the effects of melatonin (sleep period) during the night. The influence of spotlight pollution on circadian rhythms was examined in perch [54]. The data revealed that the natural nocturnal release of melatonin in water was inhibited even at the lowest light level (normal urban light pollution) but cortisol levels did not change with all illumination levels.

6. Non-circadian biochemical oscillators

Many other oscillators are found in cells that do not follow a circadian rhythm. They usually occur at higher frequencies in the range of 1-10 min (and with hearthbeat rates in the seconds range) compared to the 24-hour (1440 min) circadian cycle. They are closely associated with energy metabolism, the maintenance of the redox conditions in cells and cell division.

6.1. Glycolysis

The cyclic oscillation in nicotinamide adenine dinucleotide (NADH) levels during anaerobic glycolysis in yeasts was first discovered in the 1960s [55]. Indeed, reduced levels of NADH were shown to oscillate with periods of 3-5 min in yeast

extracts or suspensions. These oscillations were later associated with the feedback regulation of phosphofructokinase of the anaerobic glycolysis pathway. This enzyme is inhibited by high ATP/ADP ratio and activated by its product-fructose 2,6-bisphosphate. A recent study revealed that NADH oscillations in yeast could be altered by the presence of xenobiotics leading to NADH changes at higher frequencies [56]. In normal conditions, 3 h-starved yeast suspensions displayed cyclic changes in reduced NADH levels with a characteristic period between 3 to 8 min. The amplitude changes in NADH were proportional to the starvation times, whereas prolonged starvation times led to desynchronization and loss of cyclic NADH changes. Exposure to silver nanoparticles, gadolinium and copper salts lead to appearance of NADH changes at higher frequencies with concentration-dependent amplitude changes. This study revealed that biochemical oscillations could also be disrupted by environmental contaminants. Glycolytic oscillations of intact yeast cells occur at the individual cell level and could synchronize between cells as well when a threshold cell density is reached [57]. At cell concentrations below 1 x 10^6 cells/mL, each cell oscillates at its own rate (phase), which could abolish NADH oscillations at the yeast suspension level given that cell-to-cell communications are lost (desynchronized) at low density. The oscillatory behaviour of anaerobic glycolysis has led to investigations to determine whether aerobic glycolysis in mitochondria could behave in a wave-like manner.

6.2. Mitochondria oscillator

Oscillations in NADH were also found during aerobic respiration in mitochondria [58]. During respiration, various metabolites and ions, such as NADH, K^+, H^+, citrate and α-ketoglutarate, oscillate in time with a period of 2-3 min [59]. Mitochondria could even form dissipative structures in the unstirred layer of oscillating mitochondrial suspensions than can be readily measured by absorbance at 540 nm [60]. The addition of respiratory chain inhibitors suppressed these structures. Mitochondrial oscillations in NADH and intermediary metabolism precursors involve key enzymes that are regulated by free calcium (Ca^{2+}). Indeed, Ca^{2+} stimulates mitochondrial oxidative metabolism, which controls cellular ATP metabolism [61]. Fluxes of Ca^{2+}, which also exhibit oscillatory behaviour, in mitochondria activates key Ca^{2+}-dependent enzymes of the citric acid cycle, such as FAD-glycerol phosphate dehydrogenase, pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, by lowering the enzyme affinity constant (K_M) for their respective substrates [62]. This leads to the activation of mitochondrial oxidative phosphorylation for NADH and ATP production in cells. Interestingly, this activity is also regulated by circadian pathways [63]. The majority of cycling mitochondrial proteins peaked during the first hours in light where enzymes that process lipids and sugars accumulate in a diurnal manner and are dependent on the clock proteins PER1 and PER2. In mouse lacking PER1 and PER2 clock proteins, the diurnal regulation of mitochondrial respiration was lost. Hence mitochondria could display many oscillations at different frequencies: from seconds to minutes for metabolism and Ca^{2+} to 12-24 h diurnal cycles. The influence of contaminants on mitochondrial oscillation is not well understood at this time.

6.3. Calcium waves

The concentration of free intracellular calcium (Ca_i^{2+}) in the cytosol is known to oscillate in cells. This Ca_i^{2+} signaling system originates in the endoplasmic reticulum where observable Ca^{2+} waves are formed and travel to the mitochondria [64]. The temporal and spatial information of Ca_i^{2+} fluxes in cells controls many processes such as secretion, gene expression, differentiation, muscular contraction, cell movement and apoptosis [61, 65]. Transient increase in Ca_i^{2+} manifests at periods ranging from seconds to min (usually within 10-20 sec), thus at frequencies higher than the other oscillators. Calcium is released from the endoplasmic reticulum to the cytosol. These oscillations depend on inositol 1,3,5-triphosphate (IP3) which can activate calcium channels in the endoplasmic reticulum. The released Ca_i^{2+} could be transported back into the endoplasmic reticulum and removed from the cells by pumps or calcium exchangers, stimulating aerobic glycolysis in mitochondria. Indeed, calcium waves could reach mitochondria and activate key calcium sensitive mitochondrial dehydrogenases,

as described above, involving aerobic ATP-production [66]. Again, the effect of contaminants on the formation and propagation of Ca_i^{2+} waves is not well understood at this time.

6.4. Peroxidase

The first enzyme that was recognized to behave chaotically, i.e., in a non-linear and cyclic fashion, was the peroxidase-NADH-oxygen oscillator [67]. Peroxidases normally use H_2O_2 to oxidize a receiving compound (RH) leading to R-OH and H_2O. This enzyme also exhibits the capacity to use O_2 instead of H_2O_2 to remove electrons from NADH and its activity displays a cyclic (oscillatory) behaviour. This reaction is called the peroxidase-oxidase reaction: $2NADH + O_2 + 2H^+ \rightarrow 2NAD + 2H_2O$. The levels of NADH oscillate in time with a period of 1.5-2 min as a function of dissolved O_2 in a continuously stirred open reactor where O_2 is continuously introduced in the system. A characteristic absorbance at 418 nm is associated to an enzyme intermediate (compound III) which is the oxidized state of the enzyme. This state also oscillates with NADH levels (in phase), which is associated with the oxidized state of the hemic reaction centre. The frequency of the oscillations was related to the concentration of peroxidase (myeloperoxidases in neutrophils or horseradish peroxidases), with the frequency increasing with lower enzyme concentration [68]. It is therefore conceivable that the inactivation of this enzyme could lead to changes in NADH levels at higher frequencies than the normal frequency during respiration. Increasing concentration of melatonin, which follows a circadian cycle, could increase the frequency of the peroxidase-oxidase reaction leading to change in NADH levels. The frequency of the reaction increases proportionally with melatonin concentrations between 0-200 µM, and melatonin increases the pH range of oscillations from 5 to 6. The formation of the peroxidase intermediate compound III (an oxidized state of the reaction centre of the enzyme) in the presence of NADH and O_2 decreased the frequencies of the oscillations. The peroxidase-oxidase oscillator requires the addition of 2,4-dichlorophenol and methylene blue effectors, which are thought to favour and stabilize the displacement electron radicals [69]. Oscillations could be observed in the presence of naturally-occurring tyramine and 4-aminophenol. Whereas 2,4-dichlorophenol gives rise to sustained oscillations at 40 µM, tyramine promotes damped oscillations at a concentration of 120 µM owing to the antioxidant effects of tyramine. Tryptophan, the precursor of serotonin and melatonin, is converted by compound III (an oxidized intermediate of peroxidase) to *N*-formyl-kynurenine, and melatonin is oxidized by compound III to *N*1-acetyl-*N*2-formyl-5-methoxykynuramine [70], both of which prevent the inactivation of peroxidase-oxidase activity. Given that superoxide dismutase inhibits the production of this metabolite, it was proposed that compound III could act as a secondary source of O_2 radicals or participate directly in the reaction. This suggests that the oscillatory behaviour of peroxidase towards NADH levels could be changed by oxidative stress in cells and serotonin (melatonin) neurosignaling pathways involved in many neurophysiological functions in organisms at frequencies generally much higher than those involved through circadian rhythms. However, it is not known whether Prx oxidation states which oscillate daily could also oscillate at higher frequencies as is the case with peroxidases (periods of 1-3 min). The frequency of the oscillations of the peroxidase-oxidase reaction also changes proportionally with the presence of antioxidants (electron acceptors) such as ascorbate, and this property was used to sensitively determine ascorbic acid concentration at concentrations between 0.2 to 20 µM [71].

6.5. Seasonal variation

At longer periods of time, many functions in organisms follow seasonal cycles, especially in eurythermic organisms, which depend on ambient temperature. Annual cyclic changes in temperature, which are marked in temporal zones, govern many behaviors related to reproduction, such as localization of mates, gametogenesis, fertilization and embryogenesis. All of these behaviours are governed by neuroendocrine signals which couple behaviour with the internal regulation of energy assimilation (energy reserves) and reproductive organ activity [72, 73]. In fish, increased synthesis of sex steroid hormones (testosterone and 17β-estradiol) during reproduction requires cytochrome P450s, which are also involved in biotransformation of contaminants.

During steroidogenesis, the induction of cytochrome P450 by polyaromatic hydrocarbons could be repressed by estradiol-17β [74]. Conversely, the activation of AhRs could impede the production of the egg yolk protein precursor vitellogenin, which is under the control of the estrogen receptor [75]. Moreover, the expression of MT gene for heavy metal detoxification and essential element transport could be induced by estradiol-17β in cultured rainbow trout hepatocytes [76]. Hepatic MT, which bind to essential elements (Cu and Zn), could be reduced to favor Zn mobility during vitellogenesis given that vitellogenin is a zinc-containing lipophosphoproteins. The effect of pollution on seasonal variation in reproduction and xenobiotic biotransformation is better understood in the literature, but is seldom examined at the long-term multi-year level.

7. Case study

The present case study examines the effect of cadmium on the circadian behaviour of MT level and peroxidase activity in quagga mussels. MT is known to be induced by heavy metals, such as cadmium, *via* binding to cadmium and ROS [25]. Peroxidase is also involved in the elimination of H_2O_2, which can liberate ROS. These biomarkers are also known to follow circadian cycles.

Dreissena bugensis mussels were exposed to 0 and 100 µg/L $CdCl_2$ for 7 days at 15 °C under 18 h light and 6 h dark cycle. Mussels were previously maintained in aquarium water under constant aeration and fed every morning for 3 months before the initiation of exposure. During the exposure period, mussels were fed twice (every 3rd day) prior to water renewal. Mussels were allowed to feed on 1-L suspensions of *Pseudokirchneriella subcapitata* (100 million cells/L) for 3 h in the morning. After this feeding time, mussels were removed and placed in fresh exposure media. The exposure tank contained 30 mussels in 4 L of dechlorinated and UV-treated tap water under constant aeration. After the exposure period, 4 mussels each were collected at 09:00 h, 12:00 h, 15:00 h, 18:00 h, 21:00 h and 24:00 h. The mussel weights and shell length were quickly measured and they were immediately placed at -85 °C for biomarker analysis. After thawing on ice, the soft tissues were weighed and homogenized in 100 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4, 10 µg/mL apoprotinin and 0.1 mM dithiothreitol at 4 °C using a Teflon pestle tissue grinder. The homogenate was then centrifuged at 15 000 x g for 20 min at 2 °C. The supernatant was collected for MT and peroxidase activity assessments. MT levels were determined by the silver saturation assay using non-radioactive silver [77]. The samples were diluted 1/20 in bidistilled water and directly analyzed using a graphite-furnace atomic absorption spectrometer (Agilent, USA) in the presence of the matrix modifier 1% $(NH_4)_2HPO_4$. A ratio of 15 moles of Ag/mole of MT was assumed for molluscan species. MT levels were normalized against controls at the first time of collection (09:00 h). Peroxidase activity was determined using a fluorescent methodology [78]. The supernatant (20 µL) was incubated with 1 µM 2,7-dichlorofluorescein and 100 µM H_2O_2 at pH 7.4 in 10 mM KH_2PO_4 buffer for up to 30 min. The appearance of fluorescein was determined at each 5 min interval with a fluorescent microplate reader at 485 nm and 520 nm for excitation and emission wavelengths, respectively (Biotek, Synergy 4, USA). The rate of fluorescein production was normalized with total proteins in the supernatant and against control mussels at the first time of measurement (09:00 h).

In control mussels that were not exposed to Cd, the levels of MT and peroxidase activity were determined (Figure 1). For MT levels, levels changed in a cyclic fashion over the day (ANOVA $p = 0.005$), with an acrophase at 12:00 h with a minimum at midnight (24:00 h). In the case of peroxidase activity, the acrophase was reached between 21:00 h and 24:00 h with a minimum between 12:00 h and 15:00 h. There was a marginal correlation between MT levels and peroxidase activity ($R = -0.38$; $p = 0.07$), suggesting that they were inversely related to one another to some extent. However, 2-dimensional Fourier analysis of MT and peroxidase activity revealed that they were significantly coherently related but out of phase with each other at the major frequencies (i.e., between 0.041 and 0.15). Given that MT levels were higher during the light phase of the day as is the case with PERIOD proteins in mitochondria, this coincided with optimal

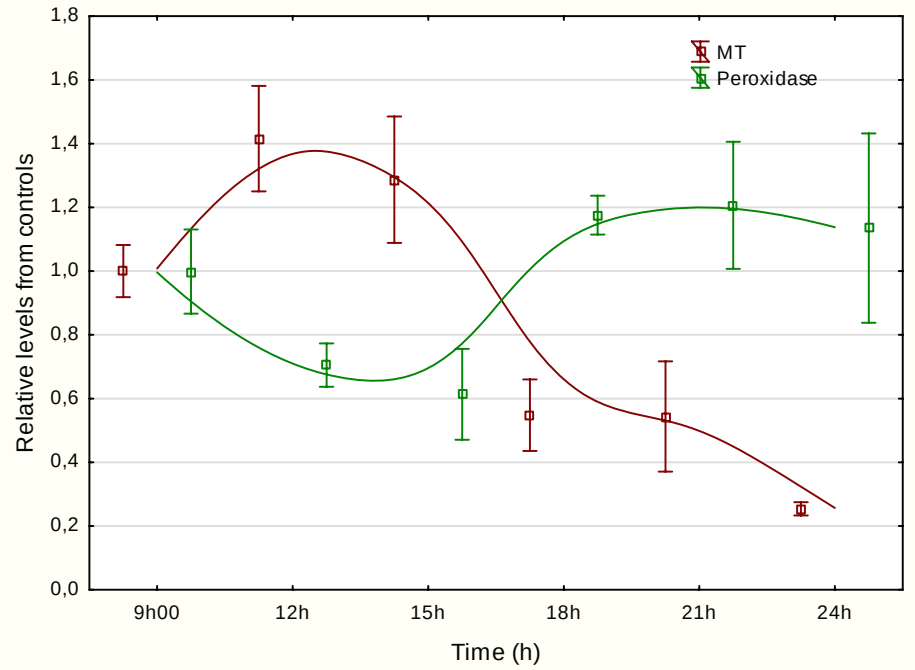

Figure 1. Changes in MT levels and peroxidase activity during the day.

The levels of MT and peroxidase activity were determined at different times in quagga mussels kept in clean laboratory water. The data represent the mean with standard error. The lines represent the least square fitting.

mitochondria activity (energy metabolism) [63]. Moreover, the diurnal regulation of mitochondrial proteins involved enzymes for carbohydrate and lipid metabolism and this regulation was absent in mice mutants lacking PER1 and PER2 proteins or mice fed with a high fat diet. The periodic changes in MT were similar to pyruvate-malate levels, which suggests carbohydrate-driven metabolism, while peroxidase activity was more closely related to lipid-driven metabolism, as determined by palmitoyl-CoA and carnithine levels. This is keeping with the release of H_2O_2 during β-oxidation in peroxisomes where increased peroxidase activity would be involved in the elimination of H_2O_2 [79].

The effect of Cd concentration on cyclic changes in MT levels were examined (Figure 2A). In mussels exposed to Cd, the data revealed that exposure to Cd lead to significant increases in MT levels as expected (Figure 2A). At 09:00 h, the levels of MT increased 4.2 times in mussels exposed to 100 µg/L Cd relative to control mussels. Correlation analysis revealed that MT levels in controls were correlated with MT levels ($R = 0.54$; $p < 0.01$), suggesting that diurnal changes are maintained when MT levels are increased in the presence of Cd. Moreover, the maximum amplitude changes were affected by Cd, which suggests that circadian control of MT levels is dampened under exposure to Cd and when Cd-detoxification mechanisms are at play (induction of MT levels). Indeed, in control mussels, MT levels were raised threefold at the acrophase compared to the lowest value at 24:00 h, while MT levels were raised 2.8-fold in mussels exposed to 100 µg/L Cd relative to controls. This suggests that exposure to Cd decreased the intensity of circadian rhythm with no apparent changes in the frequency. In trout hepatocytes exposed to a single low concentration of Cd, the MT levels were induced for the first 12 h and returned to control levels at 48 h [77]. Interestingly, the levels of calcium followed the cyclic changes in MT in cells, both of which are known to oscillate. It appears that exposure to Cd could displace the normal period of oscillations for MT and calcium in cells. In the case of peroxidase activity, no significant change in its activity was observed for mussels exposed to Cd at 09:00 h.

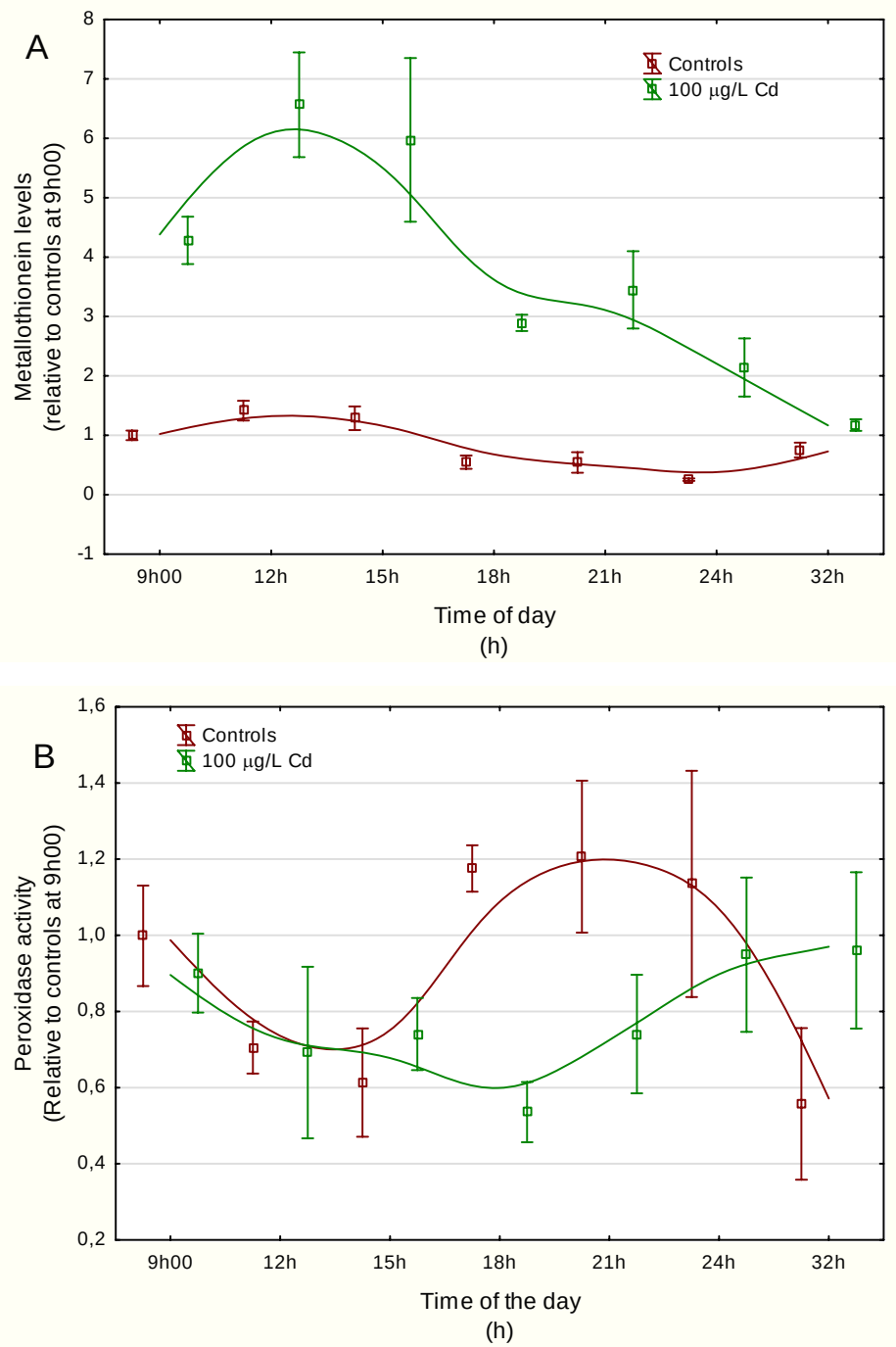

Figure 2. Diurnal change of MT levels and peroxidase activity in mussels exposed to Cd over the day.
The levels of MT (A) and peroxidase activity (B) were determined at different times during the day after exposure to Cd for 7 days. The data represent the mean with the standard error and the line represents the least square fitting.

However, peroxidase activity was significantly lower in mussels exposed to 100 µg/L at 18:00 h compared to control mussels. There was no significant correlation between peroxidase activity in control mussels and peroxidase activity in mussels treated with Cd. The cyclic change in peroxidase activity was disrupted by the presence of Cd. In mussels exposed to 100 µg/L, the

acrophase was lost compared to control mussels. It appears that the normal increase in peroxidase activity at 21:00 h is delayed by more than 24 h in mussels exposed to 100 µg/L Cd. This delayed increase in peroxidase activity could render organisms more susceptible to oxidative stress when exposed to compounds leading to oxidative stress such as Cd [80]. Sustained oxidative stress could increase oxidative damage to protein, lipids (lipofuscins, age-related pigments) and DNA, which could decrease the health status of organisms. For example, clams exhibiting increased oxidative stress and heavy metal contamination by increased lipid peroxidation and MT levels had elevated levels of age-related pigments in clams of the same age [81]. This suggests that increased oxidative stress could lead to increased physiological age as determined by increased abundance in age-related pigments. It would be of interest to determine whether clams collected at polluted sites with increased age-related pigments exhibit any signs of altered circadian rhythms.

8. Conclusions

Biochemical oscillators are found in many fundamental processes for respiration, energy metabolism and cell growth/division. On the one hand, the susceptibility of organisms to toxic chemicals could change significantly depending on the time of exposure. As observed with mice exposed to a lethal dose of cadmium, mice exposed at the beginning of the day were much more sensitive to cadmium than mice exposed later at nighttime, which became tolerant. On the other hand, circadian rhythms could be disrupted by chemicals such as cadmium (e.g., peroxidase activity and MT levels in Cd-exposed mussels) suggesting that biological clocks could be disrupted by environmental contaminants as well. The long-term consequence of such disruptions is unclear at present, but loss of synchronization with environmental cues such as light/dark, food accessibility and locomotor motor activity could be disastrous in fish in the ecological context. Indeed, even street light pollution was shown to prevent melatonin production and release in plasma in perch, which could disrupt its resting/ sleep cycles.

Other non-circadian physiological-relevant oscillators also exist in cells. From calcium waves in the cytosol and NADH waves in mitochondria, they are involved in basic and primordial functions such as cellular respiration and energy metabolism. They manifest at much higher frequencies (min) than circadian oscillators (24 h) and were also shown to be disrupted by environmental contaminants, for example the oscillatory change in NADH levels during glycolysis in yeast. Given that cells are the support for biochemical oscillations at different frequency scales, the interrelationships between them is not well understood at this time. These oscillators also provide the framework that toxic compounds may depend not only on the dosage but the time at which the exposure starts i.e, the intensity of effects and time are a continuum. In other words, there is a continuum between the effects induced by xenobiotics and time of exposure. This could explain why, in many cases, exposure concentration (dose)-effects responses are non-linear at a given exposure time point (they often display inverted U-shaped dose response curves). The understanding of the wave nature of these biochemical changes in cells could provide insights into the time-effect relationships in the initiation and manifestation of toxic action by xenobiotics.

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CONFLICT OF INTEREST STATEMENT

The author has no conflict of interest with regard to this study.

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