

**Comparing locomotor behaviour of the fish species
Danio rerio and *Leucaspilus delineatus*
under the influence of chemical stressors**

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1. Summary

This thesis is a contribution to the field of stress ecology specifically of behavioural ecotoxicology which focuses on the behaviour of animals under the influence of environmental pollutants. For analysing potential species-specific reactions to chemical stressors, two fish species were chosen: the tropical species *Danio rerio* (zebrafish) that is often used in toxicological research and the temperate species *Leucaspius delineatus* (sunbleak). Using the spontaneous locomotor behaviour of fish as a toxicological parameter, sublethal effects to the naturally occurring cyanotoxin microcystin-LR (MC-LR) and a characteristic man-made chemical 2,4,4'-trichlorobiphenyl (PCB 28) were investigated under laboratory conditions. Swimming activity of fish (motility and number of turns) was monitored continuously by using an automated video-monitoring and object-tracing system. For analysing cyclic aspects the basic behavioural analyses were combined with chronobiological procedures such as time series analysis and power spectral analysis.

Using the above methods it was shown that dissolved MC-LR concentrations between 0.5 and 50 $\mu\text{g l}^{-1}$ and PCB 28 concentrations at 100 and 150 $\mu\text{g l}^{-1}$ acted as stressors and caused significant changes in the behaviour and circadian activity rhythms of *Danio rerio* as well as *Leucaspius delineatus*. For both species elevated concentrations of the stressors led to a reduction of their activity.

Some dose-responses correspond to the hormesis theory, since there was an increase of daytime activity level at lower MC-LR concentrations and a decrease of these effects at elevated concentrations of MC-LR for both *Danio rerio* and *Leucaspius delineatus*. Furthermore for *Danio rerio* the synchronisation between activity rhythms and their zeitgeber was stronger at lower MC-LR concentrations and weaker at higher MC-LR concentrations compared to the control as indicated by the power spectral analysis.

The regression analysis between number of turns and motility was indicative for stressor-induced effects on swimming mode of fish. For *Danio rerio* the significantly decreased slope of the regression at the highest MC-LR concentration of 50 $\mu\text{g l}^{-1}$ indicated that the fish decreased the number of turns at a given motility

and swam more smoothly. However PCB 28 did not affect the swimming mode of *Danio rerio*. For *Leucaspilus delineatus* the slope of regression increased under the influence of MC-LR as well as PCB 28, indicating the fish swam more jerkily under exposure.

The data show the importance of evaluating the activity levels differentiated according to daylight and nighttime activity. So decreases of daytime activity under exposure of MC-LR and PCB 28 are, at least partly, compensated for by increases in nighttime activities for *Leucaspilus delineatus*. The same partly applies to the increase in daytime motility of *Danio rerio* at the lower MC-LR exposures that corresponded with decreases in the dark.

A degree of desynchronisation of activity to the zeitgeber light was caused by MC-LR in both fish species. For *Danio rerio* a phase delay occurred, whereby at all concentrations the changes could only be registered during the light phase. Therefore, *Danio rerio* remained diurnally active. In contrast, the phase of *Leucaspilus delineatus* advanced, whereby this shift was so drastic that a phase reverse occurred, and this species became significantly nocturnal.

Influenced by PCB 28 a degree of desynchronisation to the light/dark change which led to a phase advance, was only found for *Leucaspilus delineatus*. Both species remained diurnally active, whereby the values of the effects of zeitgeber were significantly reduced for *Danio rerio* as well as *Leucaspilus delineatus*.

A single cosinor model using a non-linear regression procedure determined the circadian rhythms of locomotor activity. In both *Danio rerio* and *Leucaspilus delineatus* cosinor analysis revealed MC-LR- and PCB 28-induced, dose-dependent alterations of the mean of oscillation (MESOR), amplitude, acrophase and period length to a different extent.

The power spectral analysis with their amplitude coefficients which gives a measure of how well the activity rhythms of fish fit infradian, circadian and ultradian sinusoidal frequencies, showed that the dominance of the circadian rhythmic peak (of 24 h) was reduced under the influence of elevated concentrations of MC-LR and PCB 28 for both species. For *Leucaspilus delineatus* simultaneously the proportion of a harmonic oscillation with a 12 h rhythm peak increased at all MC-LR concentrations.

Since the observed differences in the reactions of *Danio rerio* and *Leucaspilus delineatus* to MC-LR and PCB 28 were rather small, the results of the species *Danio*

rerio which is widely used for environmental risk assessment tests, are comparable to those of the native European species *Leucaspilus delineatus*.

The findings of this study proved that the basic behavioural analyses combined with chronobiological procedures such as time series analysis could be valuable tools for the study of stressful or even harmful environmental factors in the field of ecotoxicology as well as for biomonitoring.

1.1 Zusammenfassung

Diese Dissertation ist ein Beitrag zu dem Forschungsfeld der Stressökologie, im Speziellen der Verhaltensökotoxikologie, in der das Verhalten von Tieren unter dem Einfluss von Schadstoffen untersucht wird. Für die Analyse potentieller art-spezifischer Reaktionen auf chemische Stressoren wurden zwei verschiedene Fischarten untersucht: die häufig in toxikologischen Tests verwendete tropische Art *Danio rerio* (Zebraäbrbling) und die euroasiatische Art *Leucaspilus delineatus* (Moderlieschen). Das Verhalten wurde als Parameter zur Indikation subletaler Effekte des natürlich vorkommenden Cyanotoxins Microcystin-LR und des Xenobiotikums 2,4,4'-Trichlorobiphenyl (PCB 28) ausgewählt. Die Schwimmaktivität der Fische (Motilität und Anzahl der Wendungen) wurde kontinuierlich mit Hilfe eines automatischen Video-Monitoring- und Objektverarbeitungssystems unter Laborbedingungen aufgezeichnet. In Hinblick auf zyklische Aspekte wurden die Verhaltensanalysen mit chronobiologischen Methoden der Zeitreihenanalyse kombiniert.

Hiermit wurde gezeigt, dass MC-LR Konzentrationen zwischen 0,5 und 50 $\mu\text{g l}^{-1}$ und PCB 28 Konzentrationen von 100 und 150 $\mu\text{g l}^{-1}$ zu signifikanten Effekten auf das Verhalten und die Aktivitätsrhythmik beider Fischarten führten. Höhere Konzentrationen beider Untersuchungssubstanzen verursachten eine deutliche Aktivitätsreduzierung bei *Danio rerio* und *Leucaspilus delineatus*.

Einige der festgestellten Dosis-Wirkungsbeziehungen entsprechen der Hormesistheorie, da bei geringeren MC-LR Konzentrationen ein Aktivitätsanstieg und bei höheren MC-LR Konzentrationen ein Aktivitätsabfall beider Fischarten zu verzeichnen war. Darüber hinaus war bei *Danio rerio* die Synchronisation zwischen Aktivitätsrhythmus und Zeitgebern bei geringeren MC-LR Konzentrationen

stärker und bei höheren MC-LR-Konzentrationen schwächer im Vergleich zur Kontrolle, indiziert mit Hilfe der Power Spektral Analyse.

Die Regressionsanalyse zwischen der Anzahl an Wendungen und der Motilität eignete sich zur Analyse der Schwimmuster. Bei *Danio rerio* wurde unter dem Einfluss der höchsten Konzentration von MC-LR ein signifikant geringerer Anstieg der Regressionsgeraden registriert, der anzeigte, dass die Fische die Anzahl der Wendungen bei gegebener Schwimgeschwindigkeit reduzierten und somit expositionsbedingt eine ruhigere Schwimmweise zeigten. PCB 28 beeinflusste das Schwimmuster von *Danio rerio* jedoch nicht. Bei *Leucaspilus delineatus* führten höhere Konzentrationen von MC-LR und PCB 28 zu einem Anstieg der Regressionsgeraden und indizierten eine unruhigere Schwimmweise.

Die Daten zeigen die Bedeutung der Auswertung der Aktivitätslevel unterschieden nach Tag- und Nachtaktivität. So wurde der Abfall der Aktivität während der Hellphase bei Exposition mit MC-LR und PCB 28 teilweise kompensiert durch einen Anstieg in der Aktivität während der Dunkelphase. In vergleichbarer Weise wurde der Anstieg der Aktivität während der Hellphase bei geringer MC-LR Exposition teilweise ausgeglichen mit einem Aktivitätsabfall in der Dunkelphase. Die MC-LR Exposition verringerte bei beiden Testfischarten die Synchronisation der Aktivität mit dem Zeitgeber Licht-Dunkel-Wechsel. Dies führte bei beiden Fischarten zu einer Phasenverschiebung. Bei *Danio rerio* wurden die Aktivitätspeaks zu einem späteren Zeitpunkt registriert, wobei die Tagaktivität der Fische erhalten blieb. Bei *Leucaspilus delineatus* verschoben sich die Peaks unter dem Einfluss von MC-LR zu einem früheren Zeitpunkt hin, wobei eine Phasenumkehr zu verzeichnen war und die Fische von Tag- zu Nachtaktivität wechselten. Die PCB 28 Exposition führte nur bei *Leucaspilus delineatus* zu einer Verringerung der Synchronisation mit dem Licht-Dunkel-Wechsel, der von einer Phasenverschiebung begleitet war. Beide Fischarten blieben tagaktiv, wobei der berechnete Wert für die Zeitgeberwirkung unter dem Einfluss von PCB 28 signifikant reduziert war.

Die circadianen Rhythmen der Schwimmaktivität wurden durch ein Cosinor-Modell unter Verwendung einer nichtlinearen Regression bestimmt. Die Cosinoranalyse zeigte dosis abhängige Veränderungen von MESOR (durchschnittlicher Wert der Oszillation), Amplitude, Akrophase und Periodenlänge von *Danio rerio* und *Leucaspilus delineatus* unter dem Einfluss von MC-LR und PCB 28 an.

Die Power Spektral Analyse, deren Amplitudenkoeffizienten den Anteil infradianer, circadianer und ultradianer Frequenzen am Gesamtspektrum anzeigen, indizierte für beide Fischarten unter der Einwirkung höherer MC-LR and PCB 28 Konzentrationen eine reduzierte Dominanz des circadianen Rhythmuspeaks. Bei *Leucaspilus delineatus* war gleichzeitig ein Anstieg einer Oszillation mit einem 12 Stunden Rhythmus zu verzeichnen.

Da die registrierten Unterschiede in der Reaktion von *Danio rerio* and *Leucaspilus delineatus* auf MC-LR und PCB 28 eher gering waren, sind die Ergebnisse der Fischart *Danio rerio*, die häufig in Toxizitätstests verwendet wird, mit denen der einheimischen Art *Leucaspilus delineatus* vergleichbar.

Die Ergebnisse dieser Studie belegen, dass Verhaltensuntersuchungen in Kombination mit chronobiologischen Auswertemethoden eine sensitive und zuverlässige Abschätzung des Gefährdungspotentials von Substanzen sowohl auf dem Gebiet der Ökotoxikologie als auch für Biomonitoring ermöglichen.

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3. Introduction

During their phylogenesis, all species became adapted to their distinct habitats by means of complex, long-term, self-regulation processes which enable them to react to naturally-occurring environmental changes to maintain their physiological and ecological balance. All traits and adaptive mechanisms of recent species evolved along these historical pathways driven by interacting changes of proximate and ultimate environmental factors. If these changes take place within phylogenetic short periods, the evolved adaptive mechanisms to maintain the homeostasis can fail and furthermore new mechanisms of adaptation can often not develop in an appropriate time.

Anthropogenic activity can extend the range of natural stressors, such as increasing UV-irradiation caused by substances that deplete the ozone layer or secondary effects of man-made eutrophication leading to high concentrations of algal toxins, e.g., cyanotoxins. Furthermore anthropogenic activities rapidly produce, and introduce, new substances such as xenobiotic chemicals into the environment that may act as stressors.

3.1 ***Behaviour as toxicological endpoint***

Biological systems respond to chemical stresses on various levels of aggregation, from molecule to organisms and subsequently to populations and biocoenosis. Within the hierarchy of biological organisation the behaviour is one toxicological endpoint reflecting whole organism-level effects. Behaviour is the product of the interaction(s) of an organism with its external environment. It represents the integration of underlying physiological processes with the environmental stimuli that trigger them and the evolutionary forces that have and continue to shape them (Grue et al., 2002). Adaptations of behaviour to changes in the physical or social environment are common in the animal world, either as short-term or as long-term modifications in behavioural or physiology properties (Hofmann, 2003). The resulting behavioural plasticity is the ability of a single genotype to produce more than one alternative, potentially adaptive, behaviour in response to environmental conditions (West-Eberhard, 1989). This way behaviour turns into a major regula-

tive mechanism to overcome exogenous disturbances and stabilise the endogenous milieu (Tembrock, 1987). Activities such as food acquisition, predator avoidance, prey capture, migration, and habitat preference are critical to the survival of the organism and thus the population, and commonly used as indicators of environmental stressors (Little, 2002).

External stressful factors may influence internal physiological and biochemical processes resulting in behavioural changes. Generic pathways of stressor induced behavioural changes are shown in Figure 1.

Behavioural reactions can occur if the contaminants directly impair physiological and biochemical processes, internal sensors assess a status of inherent disorder and reactive physiological processes initialise the effectors. If the contaminants are detectable by sense organs they can lead to preference-avoidance behaviour that can significantly influence the level or duration of exposure. The impact of natural and anthropogenic stressors on the avoidance behaviour of aquatic organisms was reported e.g. by Beitinger et al. (1990), Richardson et al. (2001) and Hölker and Stief (2005).

Since behaviour is the outcome of many complex developmental and physiological processes, it should provide a more comprehensive measure than one or a few biochemical or physiological parameters (Warner et al., 1966; Zala and Penn, 2004).

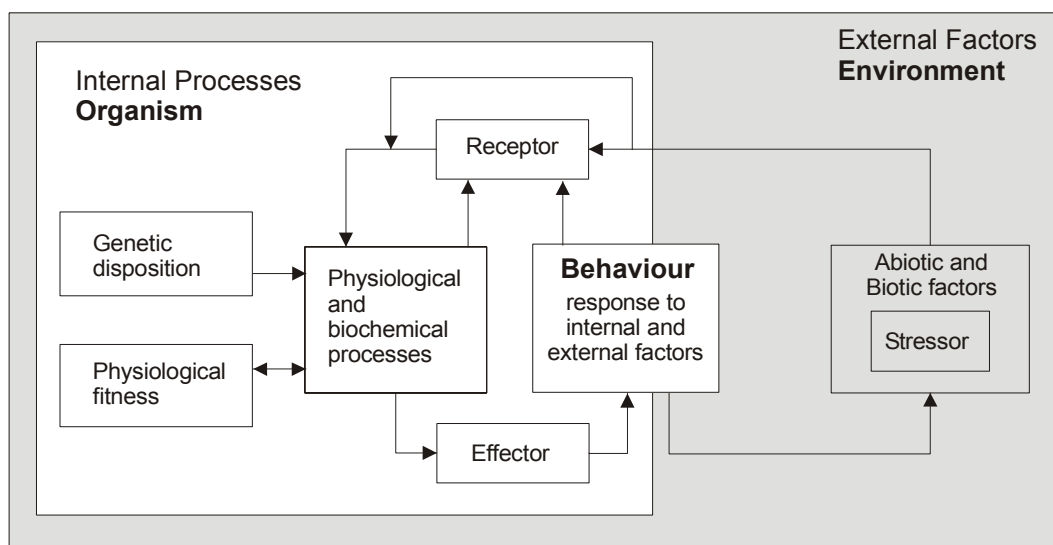


Fig. 1. The integrative role of behaviour. Behaviour is influenced by a variety of abiotic, biotic and internal factors.

A broad range of chemicals can act as behavioural toxicants through different modes of actions those are compiled in a classification scheme (Table 1) by Barron (2002). This scheme should be viewed as a generalisation of the behavioural effects and is not intended to substitute chemical-specific and species-specific evaluations of behavioural alterations. Effects of naturally occurring chemical stressors (like cyanobacteria toxins) and combined effects of pollutants are not considered therein.

Tab. 1. Classification scheme for behavioural toxicants compiled by Barron (2002).

Categories	Mode of action	Major behavioural effects
Narcotic chemicals (in the narrower sense): low molecular weight solvents e.g., alkanes, alcohols, ketones	Narcosis	Hypoactivity
Excitatory agents: halo and nitro substituted phenols and anilines	Oxidative phosphorylation uncoupling	Hyperactivity, hyperreactivity
Metals e.g., lead, cadmium, copper, mercury	Membrane damage, metabolic interaction	Feeding activity, learning, reproduction, parental care
Organometals e.g., methylmercury	Nerve tissue damage	Reproduction, parental care and learning
ChE inhibitors: organophosphate, carbamate pesticides	ChE inhibition	Hypoactivity, behavioural depression
Reactive chemicals e.g., aldehydes, alkenes, alkynes, alcohols	Electrophilic reaction with cell macromolecules	Incoordination, hyporeactivity
CNS seizure agents: organochlorine pesticides, pyrethroids	Central nervous system interaction	Incoordination, seizures, hyperreactivity, ataxia, convulsions, learning deficits
Endocrine disruptors: polycyclic aromatic compounds and other xenobiotics	Endocrine disruption	Reproductive and social behaviour

3.2 Behaviour of fish influenced by chemical stressors

In evaluating the impact of stressors in freshwater systems, fish have a special importance because they are situated at the end of the aquatic food chain, and thus

may also indicate a contamination with persistent pollutants at lower trophic levels. Fish are immersed in their physical and chemical environment, and therefore, in continuous interactions with potential environmental pollutants.

Pollutants may alter a wide variety of fish behaviours, including e.g., the sexual and reproductive behaviour, the schooling behaviour, the avoidance/preference behaviour, the chemosensory communication and the social behaviour.

Furthermore it was shown that the swimming performance of fish is affected by a range of chemical stressors including e.g., metals, CNS seizure agents, ChE inhibitors and endocrine disrupters (e.g., Spieler et al., 1977; Thomas and Rice, 1987; Reide and Siegmund, 1989; Boujard and Leatherland, 1992; Al-Kahlem et al., 1994; Steinberg et al., 1995; Saglio et al., 1996; Paul and Simonin, 1996; Spieser et al., 2000; Campbell et al., 2002; Schmidt et al., 2004).

3.3 Chronobiological aspects of behaviour

Because all behavioural reactions of organisms are essentially coupled with natural processes and physiological reactions that mainly have rhythmic components even behavioural parameters normally occur in rhythmic structures. Biological rhythms are mainly classified according to the length of the period of oscillation (τ). The rhythms whose period of oscillation is 24 ± 4 hours are defined as circadian ($\tau \sim 24\text{h}$). Rhythms with a period of less than 20 hours or more than 28 hours are defined respectively as ultradian and infradian.

Internal mechanisms of self-sustaining oscillators which generate biological rhythms of organisms (e.g., at the gene level in individual cells) are called biological clocks. A defining feature of clocks is that they can synchronise themselves using environmental time signals called time triggers (zeitgeber). Circadian clocks can be entrained by any zeitgeber that varies during a day; the dominant and therefore, physiologically most important one comes from the environmental light cycle (e.g., Aschoff et al., 1972; Pando et al., 2001; Kobayashi et al., 2003; Dekens et al., 2003).

The vertebrate clock is based on a complex hierarchy consisting of a small number of specialised central and multiple peripheral pacemakers (Schibler and Sassone-Corsi, 2002). The zebrafish *Danio rerio* is a model organism in which the molecular mechanisms of the vertebrate circadian clock have been investigated.

The zebrafish pineal gland contains both the circadian pacemaker that drives rhythms of melatonin synthesis, as well as photoreceptive molecules responsible for the entrainment of the clock phase (Cahill, 1996).

In contrast to mammals, isolated organs and cell lines from zebrafish exhibit circadian oscillations in clock gene expression that can be entrained to a 24 hours light/dark cycle (Foster et al., 2003), showing that endogenous oscillators exist in peripheral organs (Whitmore et al., 2000, Foster et al., 2003), e.g., in heart and kidney (Pando et al., 2001; Iigo et al., 2003), and in the retina (Cahill, 1996; Rajendran et al., 1996). Most peripheral cells of zebrafish (*Danio rerio*) appear to contain photoreceptors, that respond to light fluctuations in the environment by regulating oscillations in the gene expressions (Kobayashi et al., 2003). Using a cell line that derives from zebrafish embryos, differential light-dependent gene activation for several central clock components was shown by Pando et al. (2001).

Circadian clocks are involved in the persistence of circadian rhythmicity after transfer of fish from light cycles to constant conditions, these so called free running rhythms of activity were observed for several fish species including *Danio rerio* and *Leucaspius delineatus* (Hurd et al., 1998; Siegmund and Wolff, 1972). Rhythms of circadian period are evident in the locomotor activity of a number of fish species (e.g., Siegmund, 1981; Kadri et al., 1991; Siegmund and Biermann, 1992; Sims et al., 1993; Sanchez-Vazquez and Tabata, 1998; Plaut, 2000; Campbell et al., 2002). The analysis of rhythmicity allows an unspecific indication of contaminants whereby changes of the circadian rhythm in swimming activity of fish were shown for substances of different chemical categories e.g. copper, lead, formaldehyde, chlorine and chloramphenicol (Spieler et al., 1977; Steele, 1989; Reide and Siegmund, 1989; Campbell et al., 2002).

3.4 Test substances

For analysing pollutant-induced effects on fish behaviour, the cyanobacteria toxin microcystin-LR (MC-LR) and a trichlorobiphenyl (PCB 28) were investigated at sublethal levels. MC-LR was chosen as an example for a naturally occurring toxin, whereas PCB 28 was chosen as a typical xenobiotic chemical and in this respect both of them were compared. They are widespread in the aquatic environment, but there is rather few knowledge about their impact on fish behaviour.

3.4.1 Microcystin-LR (MC-LR)

3.4.1.1 Cyanotoxins

Despite the direct impact by pollutants from human usage, such as heavy metals or persistent organic pollutants, eutrophication is still the major cause of indirect deterioration of freshwater systems (Chorus, 2001). Especially under eutrophic and hypertrophic conditions cyanobacteria are known for mass developments and blooms worldwide (Paerl 1996).

Many cyanobacterial strains are known to produce a range of toxins (Codd and Poon, 1988; Carmichael, 1992), functionally classified into the acute lethal poisonous hepatotoxins and neurotoxins and the less lethal cytotoxins (Dow and Swoboda, 2000).

Microcystins are widespread cyanobacterial hepatotoxins that are produced by some cyanobacterial genera, e.g., *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc*, and *Anabaenopsis*.

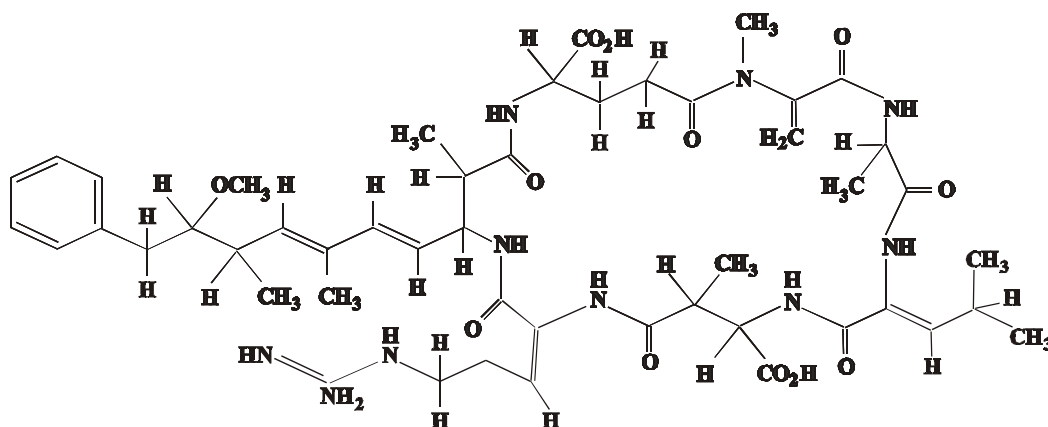


Fig. 2. Chemical structure of microcystin-LR.

Microcystins exist in more than 60 variants (Codd, 2000), and toxic strains of cyanobacteria usually produce a mixture of different microcystins (Rinehart et al., 1994; Sivonen and Jones, 1999). In this study microcystin-LR (MC-LR) was investigated which is one of the most frequently found microcystins.

Structurally, microcystins are monocyclic heptapeptides with the general structure of cyclo (-D-Ala-X-D-erythromethylaspartic acid -L-Z-Adda-D-Glu-N methyldehydroalanine-); X and Z are variable amino acids, for example microcystin-LR contains leucine (L) and arginine (R) (Fig. 2). Adda is the β -C20 amino acid (2S,

3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. The key component for biological activity appears to be linked with the Adda side chain, as cleavage of the ADDA side chain from the cyclic peptide renders both components non-toxic (Carmichael, 1992). Microcystin-LR has a molecular weight of about 1000 Daltons (WHO, 1998) and the octanol/water partitioning coefficients ($\log K_{ow}$) of MC-LR lies at 2.16 (Ward and Codd, 1999).

3.4.1.2 Concentration and persistence of MC-LR in aquatic systems

Microcystins were released into the water body mainly through the lysis of cyanobacterial cells (Watanabe et al., 1992; Sivonen and Jones, 1999).

Generally, microcystin concentrations well in excess of the WHO guideline of $1 \mu\text{g l}^{-1}$ (WHO, 1998) would be expected at sites where toxic cyanobacteria accumulate, rather than in the open water (Welker et al., 2001). Using the microcystin to chlorophyll-a ratios and the chlorophyll-a concentrations in pelagic water, the concentrations of particulate (cell-bound) microcystin in lake water were calculated by Fastner et al. (1999); in over 70% of the samples of 55 German water bodies, total particulate microcystin concentrations were below $10 \mu\text{g l}^{-1}$, however the spatial and temporal concentrations varied by 4 orders of magnitude ($2\text{--}25,000 \mu\text{g l}^{-1}$) at bathing sites during a mass development of *Microcystis* spp.. Welker et al. (2001) found the highest microcystin concentration in the lake Müggelsee (Berlin, Germany) at a site within the fringing aquatic reed belt on the downwind side of the lake; samples yielded a concentration of $120 \mu\text{g l}^{-1}$ dissolved microcystins which was, however, only a minor part (about 10%) of the total microcystins (cell-bound and dissolved) found at concentrations of up to $1,200 \mu\text{g l}^{-1}$.

Two main mechanisms for decomposition of MC-LR in water bodies are microbial activity and degradation photosensitised by natural organic matter (NOM) (Cousins et al., 1996; Welker and Steinberg, 1999, 2000; Welker et al., 2001) that may lead to a low persistence of microcystins in aquatic systems. For instance the concentration of dissolved microcystins in the lake Müggelsee (Berlin, Germany) increased from non-detectable to over $70 \mu\text{g l}^{-1}$ and dropped to non-detectable values again within a few days (Welker et al., 2001). Studies in Australia have shown that dissolved microcystins were present up to 21 days following treatment of a *Microcystin aeruginosa* bloom with an organic copper algaecide (Jones and

Orr, 1994). Currently there are no explanations for these differences in degradation time of MC-LR indicating the lack of the knowledge about underlying elimination mechanisms.

3.4.1.3 Toxicity of MC-LR

The acute hepatotoxic symptoms that result from microcystin exposure are generally caused by binding and inhibiting the serine/threonine protein phosphatase types 1 and 2A (Solter et al., 1998; MacKintosh et al., 1990, 1995). The inhibition of these enzymes results in a massive structural disorganisation of the liver cells, followed by massive internal haemorrhage, often causing mortality (Råbergh et al., 1991; Thompson and Pace, 1992; Wickstrom et al., 1996; Dawson, 1998). In addition, microcystins have been identified as potent tumour promoters (Fujiki et al., 1996; Humpage and Falconer, 1999) and were found to induce DNA damage in mouse liver (Rao and Bhattacharya, 1996).

Although it is well known that 50% of *Microcystis* blooms show hepatotoxicity (Namikoshi and Rinehart, 1996), little is known about influences of cyanobacteria on aquatic organisms. *Microcystis* caused damages of zooplankton by inhibition of population growth and feeding activity, reductions in body size, filtering rate and survival time (Jungmann and Benndorf, 1994; Smith and Gilbert, 1995; Rohrlack et al., 1999, 2001). Smith and Gilbert (1995) described the potential of *Microcystis* to alter the zooplankton community structure by differentially impacting individual species. Furthermore microcystins may also affect plants (Yamasaki, 1993; Pflugmacher et al., 1998).

The prevailing focus of investigations of the impact of cyanobacterial toxins on fish has been on the acute toxic effects of intraperitoneal injection and oral application of microcystin. The main effects of MC-LR found in these studies were damage to the liver, kidneys, or gills; disturbances of the ion balance; changes in cardiac function; growth inhibition; and mortality (Phillips et al., 1985; Sugaya et al., 1990; Tencalla et al., 1994; Råbergh et al., 1991; Carbis et al., 1997; Bury et al., 1995, 1997; Gaete et al., 1994; Rodger et al., 1994; Kotak et al., 1996; Fischer and Dietrich, 2000; Zimba et al., 2001; Zambrano and Canelo, 1996; Zhao et al., 2004; Li and Xie, 2004). The LD₅₀ of MC-LR (550 µg kg⁻¹) in common carp (*Cyprinus carpio* L.) caused total loss of the parenchymal structure of the liver and degeneration of kidney tubuli (Råbergh et al., 1991).

In contrast to studies of acute toxicity, an ecological vague toxicity parameter, only a few findings on sublethal and chronic influences of microcystin have been reported. For instance, Råbergh et al. (1991) found several types of liver damage in fish that had been injected sublethal doses of the toxin. The so called “netpen liver disease” of Atlantic salmon was also associated with microcystins (Kent et al., 1990; Andersen et al., 1993; Williams et al., 1995).

The main uptake route of microcystin in trout was the gastrointestinal tract and toxicity was manifested as massive hepatic necrosis (Tencalla et al., 1994). In contrast no microcystin related effects on the growth of *Rutilus rutilus* fed with *Aphanizomenon* and *Microcystis* were found by Kamjunke et al. (2002).

With respect to ecological issues, it is necessary to study the effects of microcystins diluted in water because aquatic organisms also may absorb the toxins directly by their body or cell surfaces. It is reasonable to assume uptake via gills because direct effects of MC-LR on gills were reported by Zambrano and Canelo (1996).

Wiegand et al. (1999) showed there was uptake of MC-LR by zebrafish (*Danio rerio*) in early life stages, whereby MC-LR accumulated in the embryo by the factor 1.2. Recent evidence indicates that fishes and invertebrates can bioaccumulate cyanotoxins, and the ingestion of contaminated food represents one clear human health risk (Magalhães et al., 2003).

3.4.2 Trichlorobiphenyl (PCB 28)

3.4.2.1 Technical use of PCBs

Polychlorinated biphenyls (PCBs) belong to persistent organic pollutants (POPs). POPs are stable, long-lived chemicals that are environmentally persistent, prone to global atmospheric transport and in some cases (which also applies for PCBs), accumulate in the food chain to levels that are potentially toxic to aquatic and terrestrial life (UNECE, 1998; Vallack et al., 1998). PCBs are human-manufactured chemicals produced during the mid-twentieth century and extensively used in a wide variety of industrial applications including hydraulic oils, solvent extenders, plasticisers, flame-retardants, lubricants, organic diluents, and dielectric fluids due to their desirable physical and chemical properties (dielectric and flame resistance, chemical and thermal stability). Unfortunately these properties of PCBs

also contribute to their ability to lead to environmental problems. Although banned from further production (in North America and western Europe in the 1970s), PCBs can be found in almost every compartment of terrestrial and aquatic ecosystems (Tanabe, 1988; Simonich and Hites, 1995). In 1977 it was estimated that approximately 68 million kg (68,000 t) of PCBs had been released into the environment, and that additional 340,000 t were still in use and a possible source of future contamination (Cohen et al., 1993).

3.4.2.2 Chemical and physical properties of PCBs

PCBs are chlorinated aromatic hydrocarbons with the general chemical formula $C_{12}H_{10-n}Cl_n$ where n is the number of chlorine atoms ranging from 1 to 10. Depending on the number and position of chlorine atoms substituted on the biphenyl moiety, 209 possible individual PCB congeners can be formed (Ballschmiter and Zell, 1980).

PCB mixtures found in the environment are different from the commercially produced PCB mixtures because of differences in physicochemical properties, persistence, and bioaccumulation among the different congeners (EPA 1999). Specific PCB products are sometimes given specific names often completed by numbers. Aroclor was the brand name for Monsanto PCBs, Phenochlor and Pyralene were brand names for PCBs manufactured by the French company Prodelec.

The log K_{ow} values of PCB congeners range from 4.5 to 8.1 (Steinberg et al., 1992). For different PCB congeners the octanol/water partitioning coefficients were directly proportional to the chlorine content of the congener (Alkhatib and Weigand, 2002). Furthermore the water solubility and vapour pressure decrease as the degree of substitution increases, and the lipid solubility increases with increasing chlorine substitution (Schwarzenbach et al., 2002).

Differences between ortho- and non-ortho substituted PCBs were found, e.g., in binding of PCBs to aquatic humic substances (Uhle et al., 1999) and in toxic effects (see 3.4.2.4). It is hypothesised that the lack of chlorine substitution at opposing ortho positions allows the two phenyl rings to rotate into the same plane (Giesy and Kannan, 2002) and so these congeners are commonly referred to as coplanar PCBs, whereas congeners with substitution in the ortho positions are referred to as non-coplanar PCBs.

In the present study the ortho-substituted congener (non-coplanar PCB) 2,4,4'-trichlorobiphenyl (PCB 28; Ballschmiter and Zell, 1980) was tested (Fig. 3, Tab. 2). PCB 28 ($C_{12}H_7Cl_3$) is one of the 6 reference congeners of the PCB/PCT/VC ordinance on the ban of PCB and PCT (1989).

Tab. 2. Physico-chemical properties of PCB 28 (from Paasivirta et al. 1999).

PCB	Configuration	Number of Cl-atoms	MW [g mol ⁻¹]	log Kow
28	2.4.4'	3	1.6×10^4	5.67

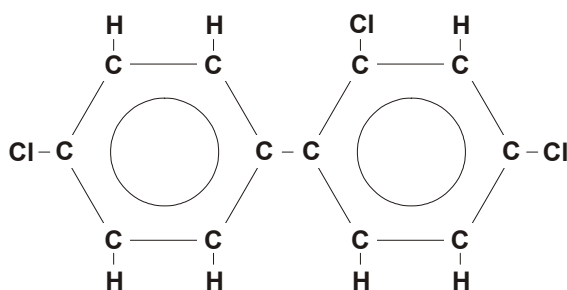


Fig. 3. Chemical structure of 2,4,4'-trichlorobiphenyl ($C_{12}H_7Cl_3$).

3.4.2.3 Concentrations in aquatic systems and bioaccumulation

The global cycling of PCBs results from their evaporation from soils and surface waters to the atmosphere and their redeposition back to land and surface water (EPA, 1999). In aquatic environments PCBs become mainly adsorbed to sediments and were found in concentrations of 9.6 to 567 $\mu\text{g g}^{-1}\text{dw}$ in freshwater sediments by Kannan et al. (1997). High PCB concentrations of 1,000 $\mu\text{g g}^{-1}$ (suspended matter) in the river Niederrhein were described by Hellmann (1986). PCBs may be mobilized from sediments if disturbed, e.g., by flooding, dredging (EPA, 1999).

Moreover the persistence of PCBs and their high lipophilicity results in their bioaccumulation in fatty tissues and their biomagnification in the food chain. Generally bioaccumulation factors increase with chlorine content from the trichlorobiphenyls up through the hexachlorobiphenyls and then generally decrease with higher chlorine content of hepta- and octachlorobiphenyls (EPA, 1999). For in-

stance bichlorobiphenyls display an approximately 450-fold decrease in the tendency to bioaccumulate in fish compared with tri- and tetrachlorinated PCBs (Abramowicz and Olson, 1995). Furthermore it was found that the chlorine atoms in position 2, 4 and 5 at least one phenyl ring of the PCB molecule were a dominant factor causing accumulation of PCBs in aquatic organisms e.g., in smelt (*Osmerus mordax*) (Gagnon et al., 1990).

There is a considerable controversy about the relative contribution from food versus direct uptake from water in determining organochlorine levels in aquatic biota (Moriarty, 1988). Bruggeman et al. (1981) described the diet as primary route by which fish accumulate PCB compounds with log K_{ow} values greater than 5, whereas according to Borlakoglu and Haegele (1991) the concentration of PCBs in fish depends primarily on the PCB concentration in the sediments and particulate matter in the ambient water. High bioconcentration factors of 28 different PCB congeners (with n ranging from 2 to 10) between 7,710 and 940,000 (wet weight) were found in zebrafish (*Danio rerio*) by Fox et al. (1994). PCBs have been detected at $\mu\text{g g}^{-1}$ levels in fish from contaminated areas which were not appreciable below the current FDA action level of $2 \mu\text{g g}^{-1}$ (e.g., Elskus et al., 1994; Stow et al., 1995).

3.4.2.4 Toxicity of PCBs

PCB exposure is associated with a wide array of acute toxic effects on fish including liver damages, impairment of osmoregulation, reduction of immune functions, reproductive dysfunctions, impairment of sexual maturation, developmental disturbances, apoptosis ATPase inhibition, altered retinoid homeostasis and mortality (Merkens and Kinter, 1971; Koch et al., 1972; Hansen et al., 1974; Nebeker et al., 1974; Svoboda et al., 1994; Monosson et al., 1994; Rice and Schlenk, 1995; Billsson et al., 1998; Kim and Cooper, 1999; Piechotta et al., 1999).

PCBs can act as endocrine-disrupting chemicals that alter the behaviour of vertebrates as reviewed by Zala and Penn (2004). Hydroxylated metabolites of PCBs (OH-PCBs) have been shown to have agonist or antagonist interactions with estrogen receptors (Carlson and Williams, 2001).

The congeners appear to act by a variety of mechanisms. One proposed mechanism is based on the high affinity of coplanar PCBs (like dioxins) for the aryl hydrocarbon receptor (AhR) where the ligand-AhR complex induces the synthesis of

the cytochrome enzyme P4501A1 (CYP 1A1) which was obtained by *in vivo* and *in vitro* experiments of fish (Hermens et al., 1990; Clemons et al., 1996; Stegeman and Lech, 1991; Monosson and Stegeman, 1991; Koponen et al., 2000). These coplanar PCBs may exert, thus, dioxin-like effects in addition to AhR independent effects which they share with non-coplanar PCBs that have no or only slight AhR agonist activity.

Based on studies that indicated the role of the AhR in mediating toxic and biochemical effects induced by PCBs, a TCDD equivalency factor (TEF) approach was developed. This approach allows the expression of toxic potential of a complex mixture of individual congeners as one integrated parameter, the toxic equivalency value, in which the toxic potency of the mixture corresponds to the potency of the most toxic congener, TCDD (2,3,7,8-tetrachloro dibenzo-*p*-dioxin). However, for every PCB congener tested, the TEF values are response- and species dependent (Safe, 1990).

Another mechanism of PCB toxicity may be related to the genetic level, e.g., the bioactivated form of a coplanar PCB led to an increase of the recombination rate in somatic cells of *Drosophila*; in contrast, PCB was not genotoxic in bacterial systems (Butterworth et al., 1995; McGowen et al., 2000).

Most of the acute toxic effects on fish are related to commercial PCBs mixtures and to coplanar PCBs. In contrast, only a few findings exist concerning effects of non-coplanar PCBs. The development of scientifically based regulations for the risk assessment of PCBs requires analytical and toxicological data on the individual PCB congeners present in any technical mixture (Giesy and Kannan, 2002), since the degree of chlorination and the chlorine positions on the molecule will greatly influence the fate and toxicity of each congener (Tang et al., 1991).

Particularly, with the recent developments in the understanding of non-dioxin-like effects of PCBs, it is pertinent to examine critically the effective doses at which non-coplanar PCBs could elicit non-dioxin-like effects in animals (Giesy and Kannan, 2002). Non-coplanar congeners may be responsible for the neurobehavioural effects of PCBs, but most of these effects were related to mammals including humans (e.g., Schanz et al., 1991; Seegal, 1996). In contrast, little information is available on behavioural toxic potencies of PCBs on aquatic organisms. For that reason in the present study the behaviour of fish was investigated under the influence of a single non-coplanar PCB congener (PCB 28).

3.5 **Aim of this study**

The main focus of this thesis is the analysis of specific behavioural aspects of two fish species under the influence of chemical stressors and therefore, a contribution to the behavioural approach for ecotoxicological issues. The chosen fish species both belonging to the family Cyprinidae were:

- a) the tropical species zebrafish *Danio rerio*,
- b) the temperate species sunbleak *Leucaspius delineatus*.

Effects of chemical stressors on *Danio rerio* that is a model system for integrative physiology and toxicology were compared with those of the native Eurasian species *Leucaspius delineatus*.

For analysing pollutant-induced effects on fish, chemicals were selected which may serve as model substances for investigating toxic effects of chemical stressors on behaviour:

- a) the naturally occurring cyanobacteria toxin microcystin-LR (MC-LR) and
- b) a typical man-made substance 2,4,4'-trichlorobiphenyl (PCB 28).

Both substances were tested on both species in different concentrations on a sub-lethal level for investigating their influences on behaviour and to answer the following questions:

Do MC-LR and PCB 28 act as stressors for fish and if they do, which stress symptoms on the behavioural and chronobiological level can be evaluated?

Do fish show similar reactions when exposed to different pollutants?

Are dose-related effects detectable and can they theoretically be described?

How consistent are the reactions among different species?

Which is the temporal development of mean motilities over the whole period of exposure to the stressors?

A second but not less important objective was to apply different analytical methods to the data derived from the tests. So the basic behavioural analyses were combined with chronobiological procedures such as cosinor analysis and power spectral analysis which are not commonly used in estimating the risks of aquatic contaminants. Questions to be answered in this respect were:

Which chronobiological methods are useful to enhance behavioural investigations in the field of ecotoxicology?

Do the analyses of rhythmical changes provide novel information on the stress potential of the investigated contaminants?

Is the application of time series investigation valuable for studying harmful environmental factors?

Do the analysis of the relation between motility versus number of turns by using regression analysis indicate exposure-induced changes in swimming mode?

Last but not least, the widening of the basic knowledge for applying behavioural tests on fish as standardized methods for biomonitoring was considered to be of practical importance and raised these questions:

How are the experiences gained in this study applicable for biomonitoring?

Which standards of the experimental design are applicable for biomonitoring using behavioural toxicity endpoints?

What importance has chronobiological aspects in the field of biomonitoring?

Furthermore the results are discussed regarding potential ecological effects of the induced changes in behavioural pattern including cycling aspects (e.g., swimming activity levels and circadian rhythms) which may lead to some adverse consequences for fish populations or communities.

4. Material and Methods

4.1 Fish species

The two fish species studied under laboratory conditions were the zebrafish *Danio rerio* and the sunbleak *Leucaspius delineatus*, both of which were obtained from laboratory stocks.

The strain of *Danio rerio* that was used, was provided by Dr. Oberemm, Institute of Freshwater Ecology and Inland Fisheries (Berlin, Germany). The breeding groups consisted of about 12 wild-type females and 15 males of *Danio rerio* each, that were derived labelled as a Singapore import, from a wholesale trader. The animals were kept under standard conditions according to Westerfield (1993) and they were 160-171 days of age at the start of each experiment.

The strain of *Leucaspius delineatus* that was used, was provided by Dr. Jäh-ninchen, Institute of Freshwater Ecology and Inland Fisheries (Berlin, Germany) and was laboratory reared offspring of a population from lake Malchower See (Germany). The animals were acclimated to a recirculation system at a temperature of 20°C and kept under standard conditions (adapted from Westerfield, 1993). They were 170- 185 days of age at the start of each experiment.

For both species, 6 schools of seven adult individuals each were kept in 15-litre glass aquaria with a swimming space of 40 x 25 x 15 cm. For *Danio rerio* one school consisted of three females and four males. For *Leucaspius delineatus* the sex ratio could not be ascertained *in vivo*. Mean total length and mean body mass of both species are shown in Table 3.

After 3 weeks of acclimatisation to the test conditions (see 4.2), the behaviour of all fish groups was recorded under standard test conditions. Thereafter, during exposure four groups of both species were exposed to the test substances MC-LR or PCB 28 (see 4.3). New groups of animals were used for every experiment.

Tab. 3. Mean total length (TL) and mean body mass (BM) of *Danio rerio* and *Leucaspis delineatus* individuals for the tests with MC-LR and PCB 28.

	<i>Danio rerio</i>		<i>Leucaspis delineatus</i>	
	TL [cm]	BM [g]	TL [cm]	BM [g]
MC-LR	3.45 ± 0.26	0.25 ± 0.02	4.55 ± 0.22	0.30 ± 0.02
PCB 28	3.47 ± 0.25	0.26 ± 0.03	4.57 ± 0.21	0.31 ± 0.03

4.2 Test conditions

The experiments aimed to maintain very constant external conditions concerning water quality parameters, artificial illumination in a distinct time regime, avoidance of optical or visual perturbations and exclusion of noise and vibrations. Feeding, temperature and artificial light/dark rhythms were constant and automatically controlled. Fish were exposed to a 12:12 h light/dark rhythm (without any natural light). Six 60 W halogen lamps were used to illuminate the aquaria during the daylight phase. The light intensity on the water surface of the aquaria was 800 Lux. Infrared light (of 880 nm) was used to illuminate the arenas during the nighttime phase. This wavelength is not detectable by the visual system of fish that is limited up to a maximum of 800 nm (Douglas and Hawryshyn, 1990). Over a period of 10 minutes the light was dimmed to become gradually on or off. The animals were automatically fed with TetraMin[®] flakes twice a day at a ratio of 3% body mass per day, three and seven hours after light-on.

Tab. 4. Physico-chemical parameter of the aquaria water for the experiments with *Danio rerio* and *Leucaspilus delineatus* under the influence of MC-LR and PCB 28.

Parameter	Unit	Measured value
Temperature	°C	26 ± 0.5 (1)
		20 ± 0.5 (2)
Conductivity	µS cm ⁻¹	720 ± 10
pH- value		7.5-7.7
Total hardness	°dH	16.5 ± 0.5
Total organic carbon (TOC)	mg l ⁻¹	4.1 ± 0.4
Oxygen	mg l ⁻¹	7.7 ± 0.2 (1)
		8.2 ± 0.3 (2)
Ammonium	mg l ⁻¹	<0.5
Iron	mg l ⁻¹	<0.03
Nitrate	mg l ⁻¹	5 ± 0.2
Nitrite	mg l ⁻¹	<0.03

(1) For the experiments with *Danio rerio*

(2) For the experiments with *Leucaspilus delineatus*

For the experiments aerated drinking water (“Berlinwasser Holding Friedrichshagen”) was used. The physico-chemical composition of the water is listed in Table 4. Basic water quality parameters of pH, oxygen and ammonium were measured both in storage tanks and aquaria once a day. The physico-chemical parameters of the used aquarium water were constant over the exposure period and within the normal physiological ranges for fish (Schreckenbach et al., 1987, 2001; Schäperclaus, 1990).

Tests were carried out in a flow-through system with a continuous discharge of 10 l aerated tap water per aquarium and day from storage tanks whereby the flow rates were controlled by multi-channel peristaltic pumps.

4.3 Test substances

4.3.1 Microcystin-LR

The test substance MC-LR was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Purified MC-LR (10 mg) was dissolved in 1 ml of methanol and diluted in 100 ml of distilled water. To keep experimental conditions as consistent as possible, fish exposed to 0.003 % methanol in water only served as controls. A comparison of behaviour during the pre-exposure standard test conditions with behaviour of the controls during exposure revealed that the single exposition to 0.003% methanol in water had no effect on behavioural parameters.

During the exposure period both fish species were exposed to four different concentrations of MC-LR (nominal concentrations): 0.5, 5 and 15 $\mu\text{g l}^{-1}$ for a period of 17 days each and 50 $\mu\text{g l}^{-1}$ for a period of 6 days. The storage tank water (10 l) was renewed daily and MC-LR was added at nominal concentrations.

4.3.2 Trichlorobiphenyl

The test substance trichlorobiphenyl was purchased from Sigma-Aldrich, Schnelldorf, Germany. PCB 28 (60 mg) was dissolved in 6 ml ethanol and diluted in 80 ml of distilled water. Fish exposed to 0.003% ethanol in water only served as controls.

During the exposure period four groups of fish were exposed to two different (nominal) concentrations of PCB 28 (duplicates): 100 and 150 $\mu\text{g l}^{-1}$ for a period of 8 days. The storage tank water was renewed daily and PCB 28 was added at nominal concentrations. Two groups served as controls, the first group without any exposure to solvents and the second group with 0.003 % ethanol. There were no significant differences between both control groups, what revealed that the single exposition to 0.003 % ethanol in water had no effect on behavioural parameters.

4.4 Recording Procedures

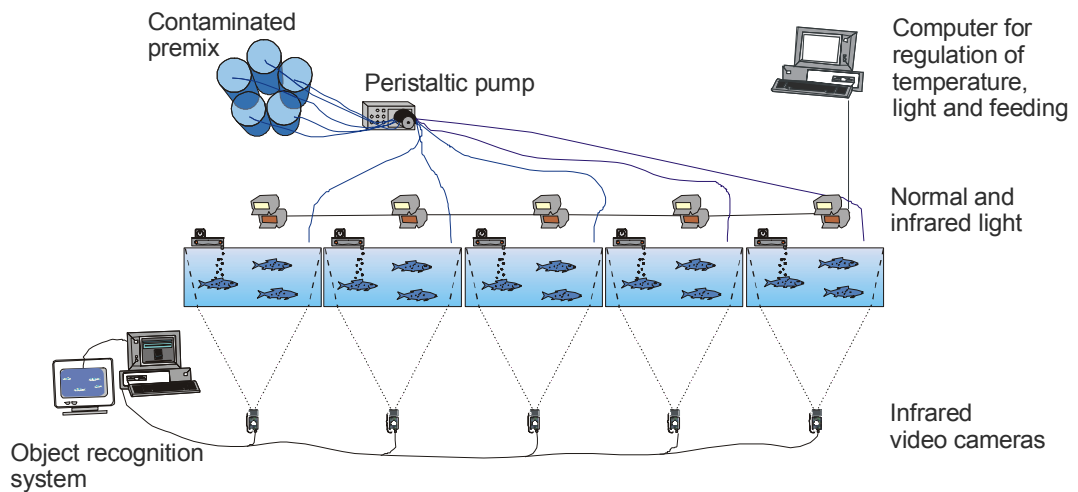


Fig. 4. Scheme of the equipment.

The fish activity was monitored continuously with the automated video processing system BehavioQuant[®] (Spieser et al., 2000). The experimental design is shown in Figure 4. Fish were observed by infrared video cameras, one in front of each tank which were able to handle normal as well as infrared light, enabling the continuous observation even during the night. The positions of the untagged fish were recorded at a two-dimensional area, data were digitised and paths of individual fish afterwards tracked by the object recognition software. Thus it was possible to reconstruct the real movements of every fish of the school.

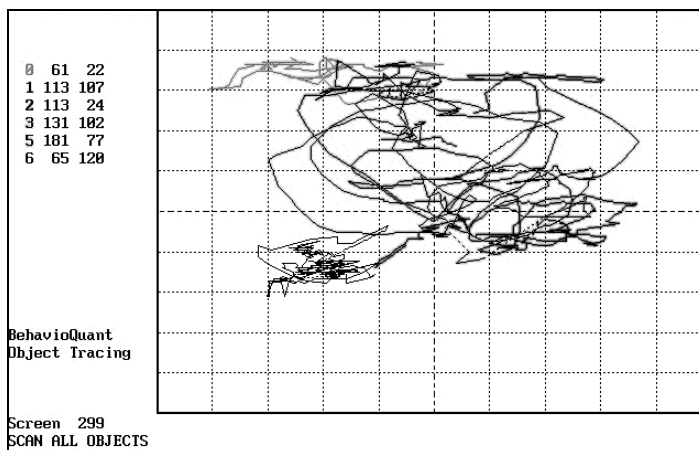


Fig. 5. Screen shot of the movement tracks of one fish group during one measuring interval of 2 minutes. The different lines represent the single individuals.

The screen shot (Fig. 5) shows an example of the movement tracks of one fish school. Video was filmed at a frequency of 25 frames per second, and overall there were 69 measuring cycles of 2 min per day. Every picture of the experimental chamber was in real time compared point-by-point with a background reference picture. The x - y positions of recognized objects are written to disk for every measuring interval. The raw data were pre-processed and converted into tables which contained the behavioural parameter values: the motility is the swimming velocity in video-pixels per second and the turns are the number of changes of the direction per second. Motility and turns characterised the swimming activity.

4.5 Statistics and calculations

4.5.1 Analysis of mean motility and mean number of turns

The spontaneous locomotor behaviour of *Danio rerio* and *Leucaspius delineatus* was registered 23 hours per day, and data were averaged per hour. Mean motility and mean number of turns were analysed in different ways:

over the whole measured time per day (23 h d⁻¹) and over the whole exposure period

divided into light and dark phases over the whole exposure period

divided into light and dark phases over intervals of the exposure period

On these bases all the results of the exposed groups were compared with those of the controls. Statistical analysis of all results was performed in SPSS 9.0. using the ANOVA procedure, as well as the Dunnett T3 post hoc test for comparison of groups with unequal variances. Generally significant differences were accepted at $p < 0.05$. In all figures and tables a significance level of

$p < 0.05$ is indicated by one asterisk,

$p < 0.01$ is indicated by two asterisks, and

$p < 0.005$ is indicated by three asterisks.

4.5.2 Regression between motility and turns

For each exposed group and for the control a regression between motility versus number of turns was fitted. A linear model ($y = a \cdot x + b$) proved to be most appropriate for the totality of evaluated relations. F-statistics were performed for testing the significance of the determination coefficients r^2 . Student's t -tests were used to

compare the values for slope and intercept to 0.0. Deviations between regression parameters of control and exposition in detail were evaluated by calculating and comparing their 95% confidence intervals.

4.5.3 Zeitgeber analysis

Effects of zeitgeber (time trigger) that were calculated as a quotient of the motility during the light phase and the overall motility during the light and dark phase allows one to distinguish between diurnal and nocturnal activity rhythms of the test species. Values between 0 and 0.5 indicated that the animals were nocturnally active and values between 0.5 and 1 that they were diurnally active. Statistical evaluation was made by the Student's *t*-Test to compare the group values to 0.5 and furthermore the exposed group to controls using the computer program SPSS 9.0.

4.5.4 Cosinor analysis

Oscillations are normally characterized by up and down, left and right or back and forth movements of measurable parameters (*y*-values) during the course of time (*x*-values). So they appear in a diagram as wavelike or vibration or pendulation curves.

These curves are mathematically modelled by trigonometric functions like sinus or cosinus which are adapted to the exact shape of the curve by multiplicative and additive terms for example: $y = A * \sin (B * t + C)$ (*t* being time, *A, B, C* being variables).

In the present study circadian rhythms of locomotory activity (represented by the motility) were objectively determined by a single cosinor model using the non-linear regression procedure of the statistical program SPSS 9.0. The implementation of this method applied a simple parameterised cosinus equation to the raw data series and used an approximation by sequential quadratic optimisation:

$$f(x) = M + A * \cos (6.283/P * (x - K)).$$

The variables are explained in Figure 6.

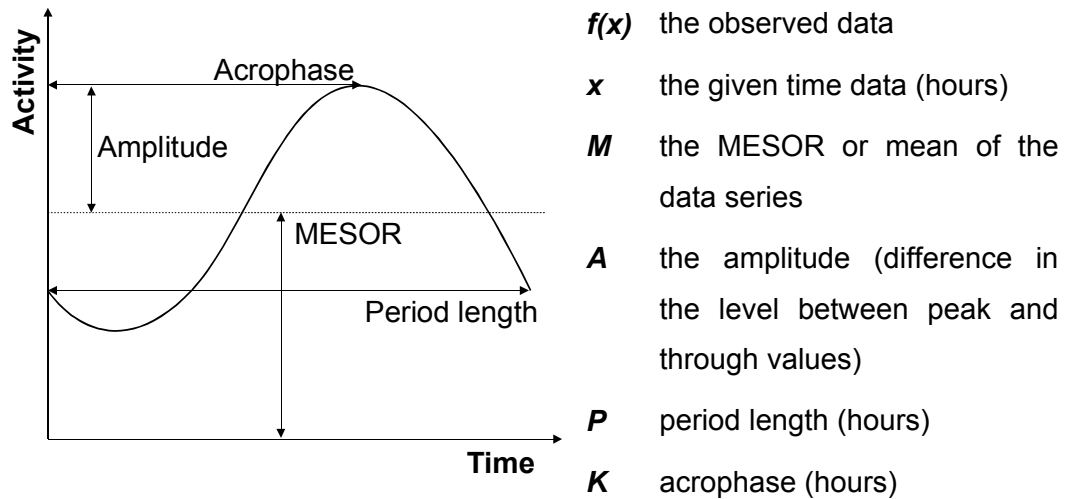


Fig. 6. Scheme of a circadian rhythm.

The acrophase is defined as phase angle corresponding to the maximal value of the rhythmic parameter studied, in the present study this was the time between local midnight and maximum peak of activity. The term inside the cosinus brackets was transformed to radians by the factor $2 \cdot \pi$ (equals 6.283) so the original time units could be used inside the equation. Calculated regression parameters were considered significant if its 95% confidence interval did not include zero.

The calculated overall solutions for exposure and control were compared by an F-test (Zar, 1996). Deviation between regression parameters of control and exposition in detail was evaluated by calculating and comparing their 95% confidence intervals.

Polar Plots of the cosinor analysis visualise the acrophase and the amplitude with confidence intervals. The maximum value of activity is located with a 95% probability in this area of confidence. The radial length of vector is proportional to the amplitude, while its angular direction indicates the temporal localisation of acrophase in physical time.

4.5.5 Periodic frequency analysis

For quantification of the harmonic frequency structure of activity rhythms of *Danio rerio* and *Leucaspius delineatus* a power spectral analysis of the motility which is a Fourier transformed autocorrelation function was used. The calculations were performed by the mean of the program "Zeit" which application is described in Scheibe et al. (1999, 2002). Periodic frequencies which explain a sig-

nificant proportion of the total variation of the original data series and which are furthermore harmonic to the circadian period were ascertained. Periods are called harmonic in a chronobiological context if their lengths are integer dividers of 24 h. All periods of the power spectra were tested for significance by the integrated function of the program “Zeit” (see Scheibe et al., 1999, 2002).

Degrees of Functional Coupling (DFCs) were used for comparison of the rhythmic structures (Sinz and Scheibe, 1976; Scheibe et al., 1999). DFCs express the percentage of the circadian component and harmonic ultradian components in relation to all significant rhythmic components of a spectrum based on the respective relative parts of their assigned variance. Therefore, the DFC describes the percentage of cyclic activity components which is synchronised with the circadian rhythm.

The equation for the DFC is: $DFC=100*SP(harm)/SP(total)$ with:

SP(total) being the sum of all significant periodogram ordinates (i.e. the variance assigned to significant periods) and

SP(harm) giving the sum of those periodogram ordinates which are significant and harmonic to the circadian period.

The harmonic portion (HP) describes in contrast the percentage of the number of the circadian and their harmonic ultradian components in relation to the number of all significant periods smaller or equal to the circadian rhythm.

5. Results

5.1 Effects of Microcystin-LR

5.1.1 Mean motility over the whole exposure period

A rapid decrease of mean motility of *Danio rerio* as well as of *Leucaspilus delineatus* was noted at the highest MC-LR concentration of $50 \mu\text{g l}^{-1}$ which was statistically significant (Fig. 7). In contrast, lower MC-LR concentrations ($5 \mu\text{g l}^{-1}$, $15 \mu\text{g l}^{-1}$) tended to increase the motility of *Danio rerio* but this was not statistical relevant. However, the overall motility of *Leucaspilus delineatus* increased significantly at concentrations of $0.5 \mu\text{g l}^{-1}$, $5 \mu\text{g l}^{-1}$ and $15 \mu\text{g l}^{-1}$.

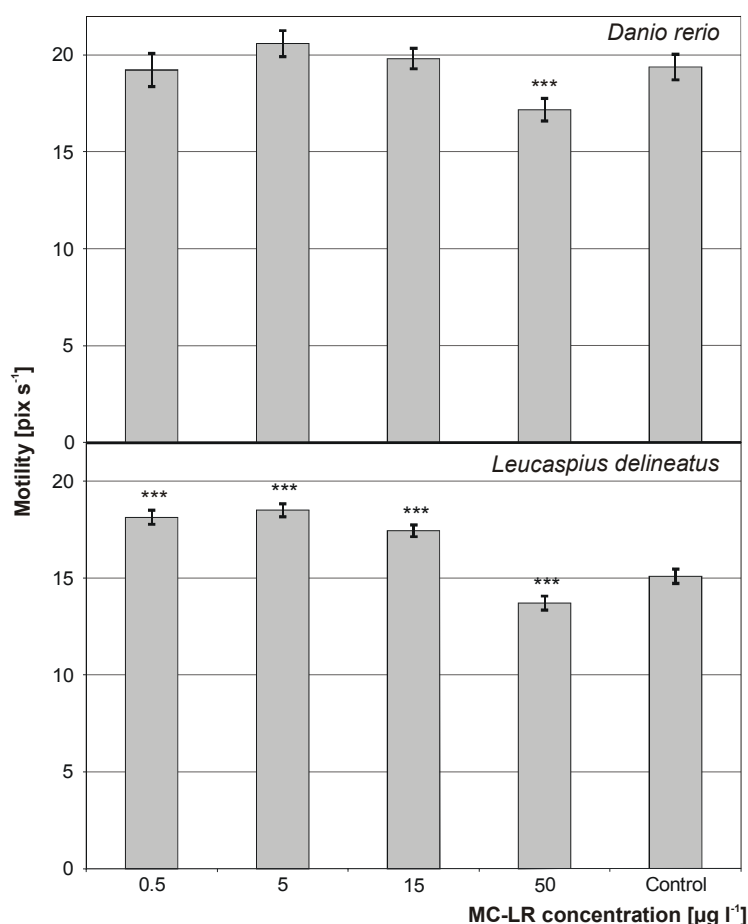


Fig. 7. Average motility of *Danio rerio* and *Leucaspilus delineatus* exposed to MC-LR over the whole measuring time per day (23 h per day) and the whole exposure period. Means and 95% confidence intervals are shown.

5.1.2 Mean motility during light and dark phases

With *Danio rerio* the lowest MC-LR exposure concentration of $0.5 \mu\text{g l}^{-1}$ led to a significant increase in motility during the light phases and a significant decrease during the dark phases. Elevated MC-LR exposure ($50 \mu\text{g l}^{-1}$) led to significantly decreased motility during the light as well as dark phase (Fig. 8). The overall influence of MC-LR is also illustrated by the daily activity curves (Fig. 9). It is shown that the significant MC-LR induced changes of activity of *Danio rerio* occurred especially shortly after the onset of light which is the period of mating and spawning behaviour for *Danio rerio*. The changes in the time interval of three hours after the onset of light were statistically significant for the lowest test concentration of $0.5 \mu\text{g l}^{-1}$ which led to an increase of motility and for the highest MC-LR concentration of $50 \mu\text{g l}^{-1}$ which led to a decrease of motility (Fig. 10).

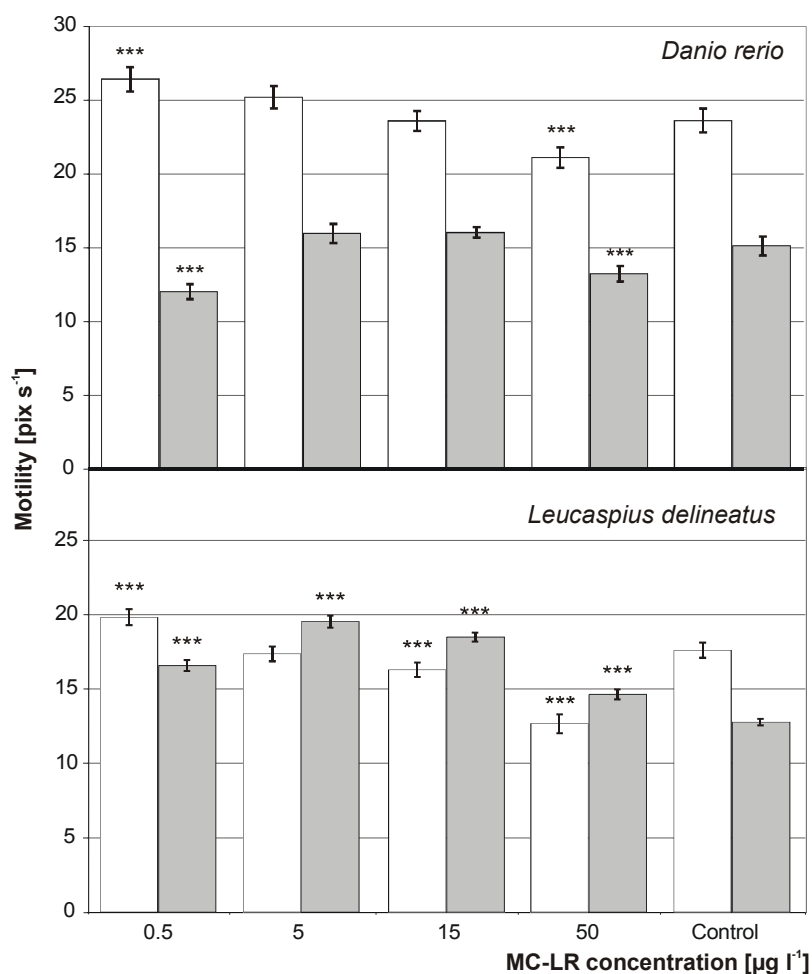


Fig. 8. Average motility of *Danio rerio* and *Leucaspilus delineatus* divided in light (white bars) and dark phases (grey bars) over the whole period of exposure to MC-LR. Means and 95% confidence intervals are shown.

With *Leucaspius delineatus* the lowest exposure concentration of $0.5 \mu\text{g l}^{-1}$ caused a significant increase in motility during both the light and dark phase. During the dark phases, the motility of *Leucaspius delineatus* also increased significantly at $5 \mu\text{g l}^{-1}$ and $15 \mu\text{g l}^{-1}$ (Fig. 8).

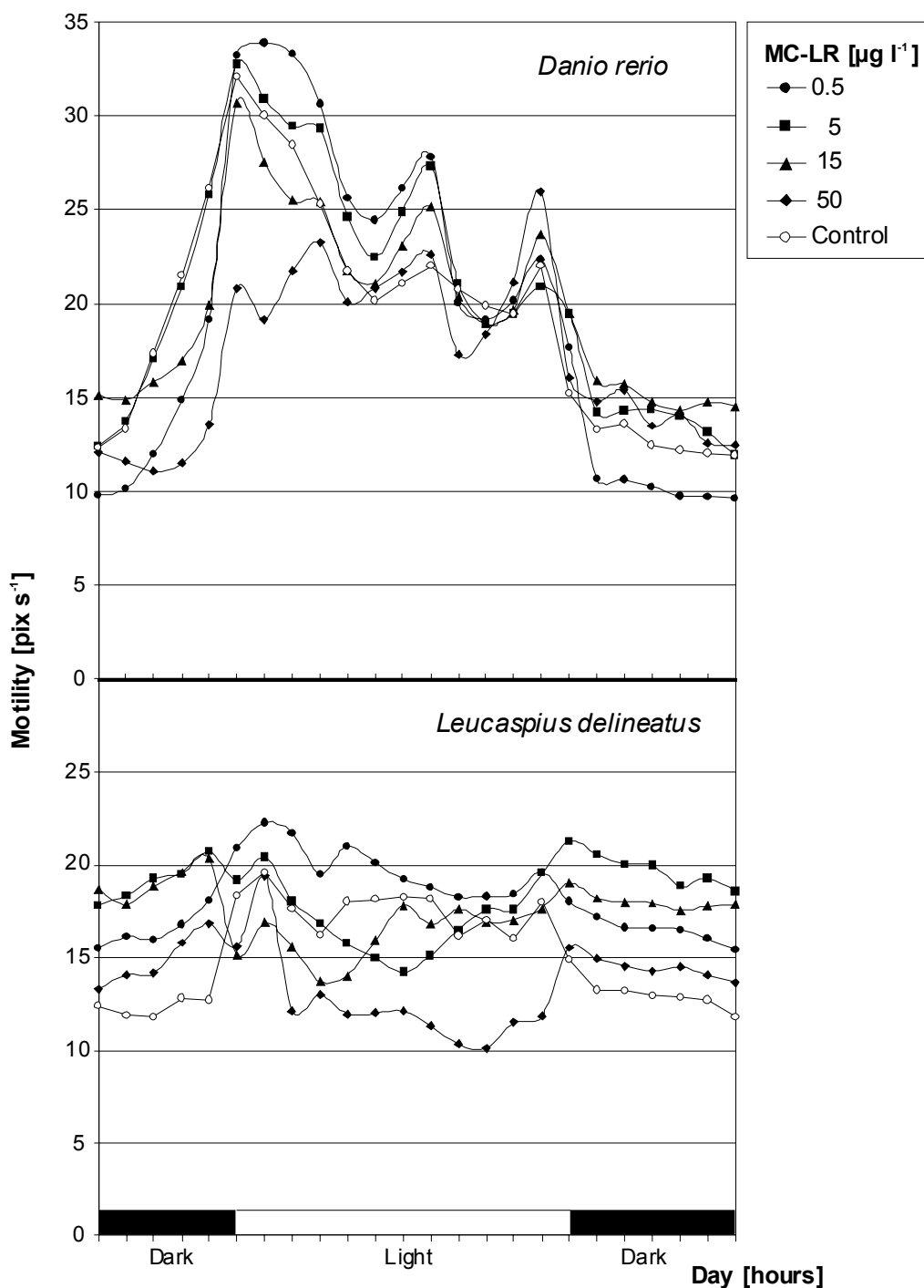


Fig. 9. Smoothed curve of average motility of *Danio rerio* and *Leucaspius delineatus* over the whole period of exposure to MC-LR.

The concentration of $15 \mu\text{g l}^{-1}$ led to a significant decrease of motility during light phases. At the highest concentration ($50 \mu\text{g l}^{-1}$) the motility during light phases decreased significantly, as was shown for *Danio rerio*. In contrast to *Danio rerio*, the motility of *Leucaspius delineatus* increased significantly during dark phases. Also for *Leucaspius delineatus* it is shown that significant MC-LR induced changes of activity occurred shortly after the onset of light (Fig. 9).

The changes in this time interval of three hours after the onset of light were statistically significant for the lowest test concentration of $0.5 \mu\text{g l}^{-1}$ which led to an increase of motility and for the higher MC-LR concentration of 15 and $50 \mu\text{g l}^{-1}$ which led to a decrease of motility (Fig. 10).

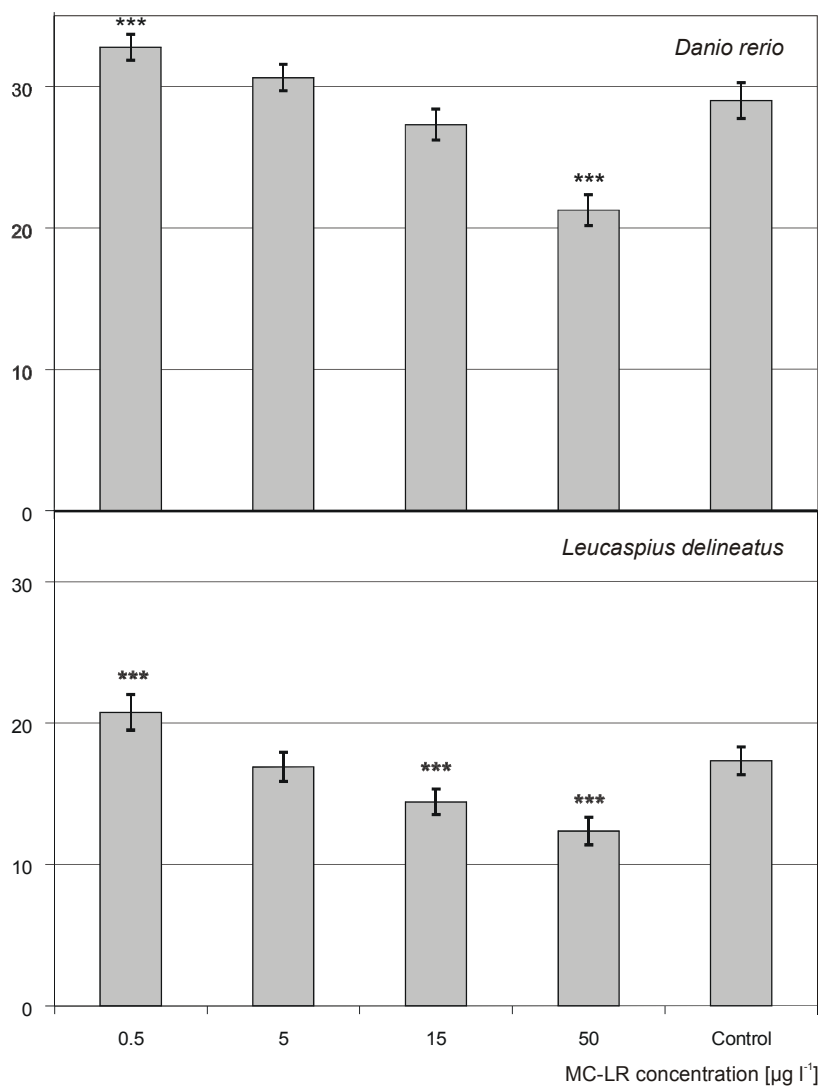


Fig. 10. Average motility of *Danio rerio* and *Leucaspius delineatus* during the time interval of 3 hours after the onset of light over the whole period of exposure to MC-LR. Means and 95% confidence intervals are shown.

5.1.3 Temporal development of mean motilities

For analysing the temporal development of mean motilities the whole period of exposure to MC-LR (17 days) was divided into five time intervals of three days each and one interval of two days. The mean motility of every exposure group was calculated for each of the 6 intervals, distinguished between daylight and nighttime activity. With *Danio rerio* during light phases, the observed increase in motility caused by the lowest concentrations ($0.5 \mu\text{g l}^{-1}$) occurred from the fourth exposure interval on and did not disappear up to the last exposure interval (Fig. 11). During dark phases, the motility at the exposure concentration of $0.5 \mu\text{g l}^{-1}$ decreased significantly already in the second exposure interval whereas significant changes did not exist in the last exposure interval. During the light phase MC-LR in a concentration of $5 \mu\text{g l}^{-1}$ caused also an increase in motility from the fourth exposure interval on which continued up to the last exposure interval, whereas during the dark phase there were only significant differences in the fourth exposure period. During the light phases, the MC-LR exposure with $15 \mu\text{g l}^{-1}$ led to a decrease of motility in the second and third interval followed by an increase of motility in the fourth and fifth interval. During the dark phases, a significant change of motility at the exposure concentration of $15 \mu\text{g l}^{-1}$ occurred only in the last interval, whereby the motility increased significantly. The highest MC-LR concentration ($50 \mu\text{g l}^{-1}$) caused a significant decrease in motility in the second and third exposure interval during the light phases and a significant decrease of motility in the first and second time interval during the dark phases. After MC-LR exposure (with $50 \mu\text{g l}^{-1}$ the exposure was only 6 days, see 4.3.1), motility returned to control values in the fourth exposure interval.

During the light phases, the motility of *Leucaspius delineatus* at the concentration of $0.5 \mu\text{g l}^{-1}$ significantly increased from the third exposure interval up to the last interval, whereas during the dark phases the motility significantly increased over all exposure intervals (Fig. 11). The MC-LR exposure concentration of $5 \mu\text{g l}^{-1}$ led to a significant increase of daytime motility only in the fifth exposure interval, whereas the motility during the night was significantly increased over the whole exposure time. The MC-LR exposure concentration of $15 \mu\text{g l}^{-1}$ caused a significant increase in motility during the dark phases from the first to the fifth exposure interval, while during the light phases the motility was not significantly changed.

At the highest exposure concentration (50 $\mu\text{g l}^{-1}$), the motility significantly decreased from the second to the fifth exposure interval during light phases, whereas

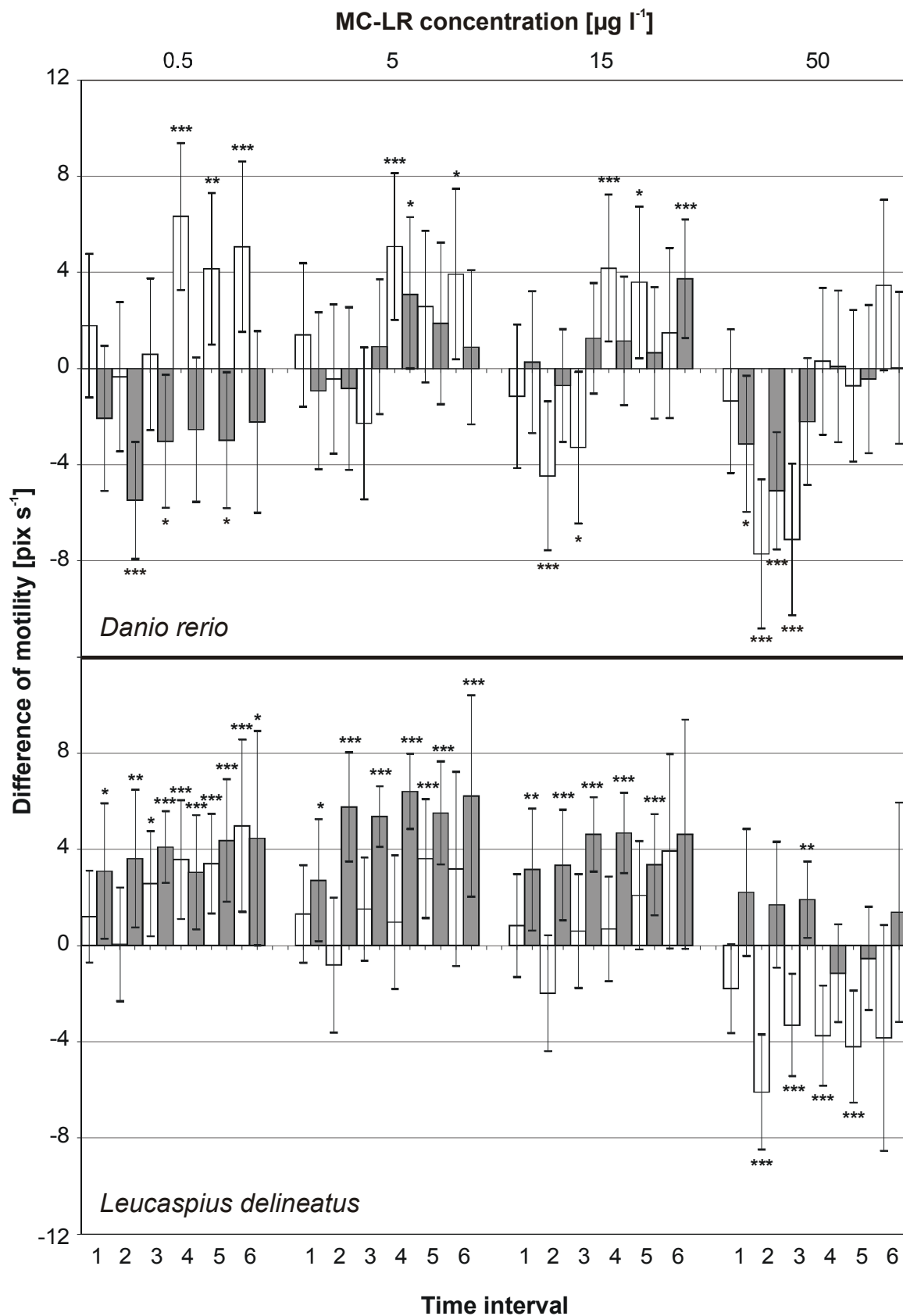


Fig. 11. Differences in motility between the different exposure groups and the con-

trols for *Danio rerio* and *Leucaspius delineatus*, divided into light (white bars) and dark phases (grey bars). The whole period of exposure to MC-LR was divided into five time intervals of 3 days each and one interval of two days. Means and 95% confidence intervals are shown.

during the dark phases the motility significantly increased only in the third time interval. At the highest exposure concentration which was terminated after 6 days, motility values returned to control levels during the last exposure interval.

5.1.4 Number of turns over the whole exposure period

With *Danio rerio*, the number of turns was significantly decreased at the highest MC-LR concentration ($50 \mu\text{g l}^{-1}$) during light phases, whereas during dark phases they rose statistically significant at concentrations of $5 \mu\text{g l}^{-1}$, $15 \mu\text{g l}^{-1}$ and $50 \mu\text{g l}^{-1}$ (Fig. 12).

With *Leucaspius delineatus*, the number of turns increased at concentrations between $0.5 \mu\text{g l}^{-1}$, $5 \mu\text{g l}^{-1}$ and $15 \mu\text{g l}^{-1}$ during light phases. In contrast to this increase, the highest exposure concentration ($50 \mu\text{g l}^{-1}$) caused a significant decrease of the number of turns. During the dark phases, the number of turns of *Leucaspius delineatus* was significantly increased at all exposure concentrations (Fig. 12).

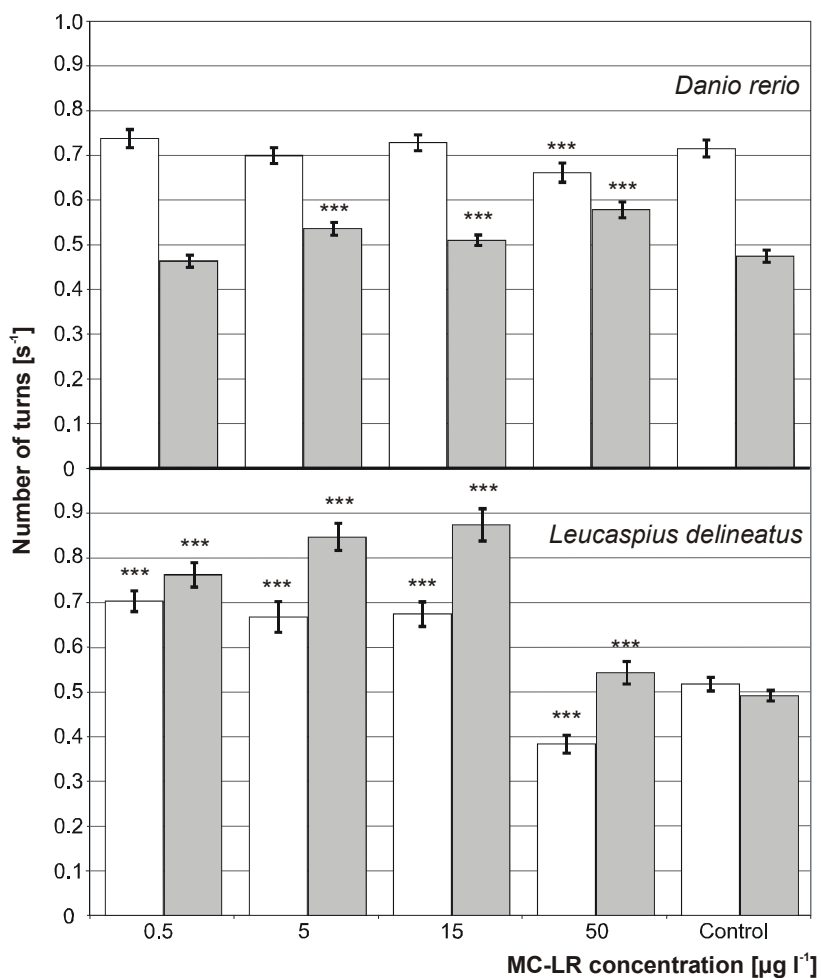


Fig. 12. Average number of turns of *Danio rerio* and *Leucaspis delineatus* divided in light (white bars) and dark phases (grey bars) over the whole period of exposure to MC-LR. Means and 95% confidence intervals are shown.

5.1.5 Regression between motilities and turns

The swimming mode of both species was analysed by regression between motility and turns. The relationship between motility and number of turns was best described by a linear function for both fish species (Tab. 5; Fig. 13).

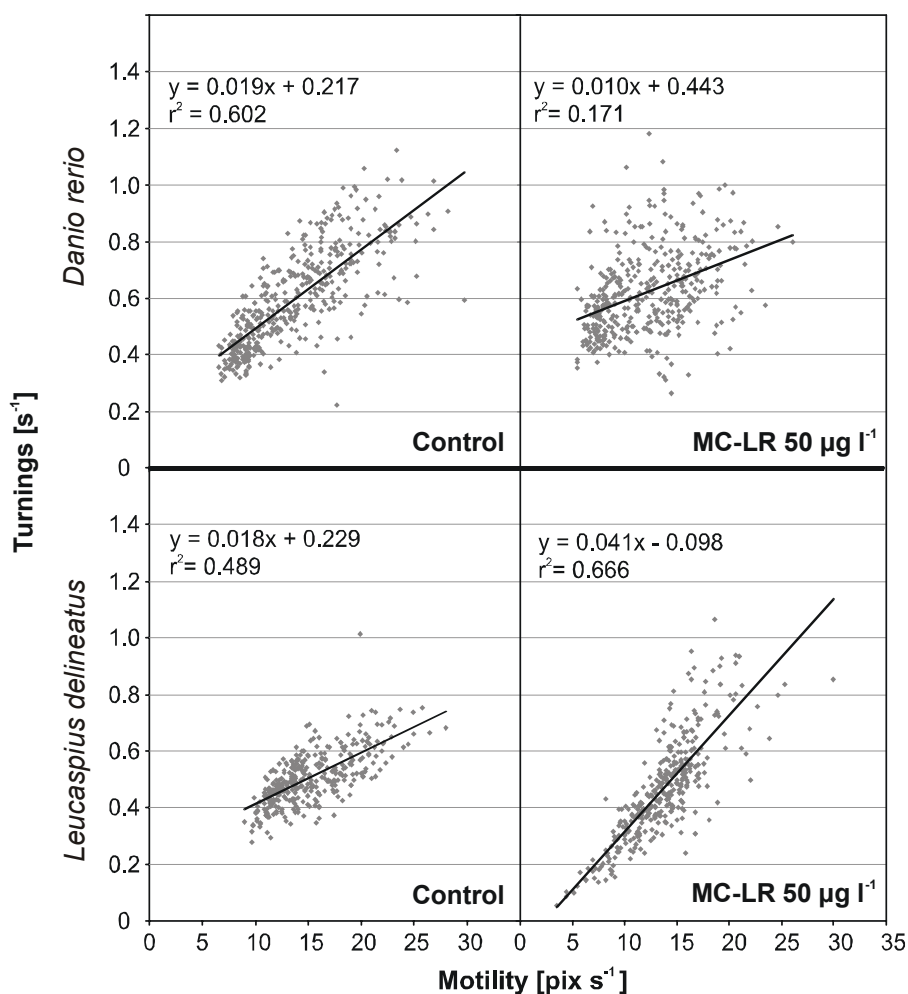


Fig. 13. Number of turns over motility with fitted linear function: $y = a \cdot x + b$ for *Danio rerio* and *Leucaspilus delineatus* exposed by MC-LR.

For *Danio rerio* under unexposed conditions, the number of turns increased with increasing motility with $r^2 = 0.602$ as determination coefficient. Elevated concentrations of MC-LR led to a decrease of the overall determination coefficient compared to the control for *Danio rerio*. At the highest MC-LR concentration of $50 \mu\text{g l}^{-1}$ a determination coefficient of $r^2 = 0.171$ was found. Although this regression was still statistically significant ($F = 83.7$; $p < 0.005$) this fact indicated that the statistic relation between the increasing number of turns and increasing motility was weaker under exposure for *Danio rerio*. Furthermore the slope of the regression was significantly decreased at the highest MC-LR concentration compared to the control.

For *Leucaspilus delineatus* under unexposed conditions, with increasing motility, number of turns increased with a determination coefficient of $r^2 = 0.489$ which

was statistically significant (Tab. 5; Fig. 13). In contrast to *Danio rerio* elevated concentrations of MC-LR led to an increase of the overall determination coefficient compared to the control for *Leucaspilus delineatus* indicating that the statistic relation between increase of number of turns and increasing motility was stronger under exposure. In contrast to *Danio rerio* the exposure to MC-LR (0.5, 5, 15 and 50 $\mu\text{g l}^{-1}$) led to significant increased slopes of the regressions.

Tab. 5. Number of turns versus motility with fitted linear function: $y = a \cdot x + b$ for *Danio rerio* and *Leucaspilus delineatus* exposed by MC-LR; Variables of the function: a (slope), b (intercept), r^2 (determination coefficient).

Species	Parameter	MC-LR concentration ($\mu\text{g l}^{-1}$)				
		0.5	5	15	50	Control
<i>Danio rerio</i>	a (slope)	0.018	0.016	0.022	0.010	0.019
	p-value	***	***	***	***	***
	b (intercept)	0.259	0.294	0.192	0.443	0.217
	p-value	***	***	***	***	***
	r^2	0.696	0.566	0.578	0.171	0.602
	F-value	928.105	528.886	556.516	83.702	612.829
	p-value	***	***	***	***	***
<i>Leucaspilus delineatus</i>	a (slope)	0.027	0.059	0.062	0.041	0.018
	p-value	***	***	***	***	***
	b (intercept)	0.250	-0.336	-0.302	-0.098	0.229
	p-value	***	***	***	***	***
	r^2	0.276	0.657	0.563	0.666	0.489
	F-value	139.284	702.415	471.898	730.570	349.551
	p-value	***	***	***	***	***

5.1.6 Effects of zeitgeber

Both *Danio rerio* and *Leucaspilus delineatus* revealed a significant diurnal activity under unexposed conditions, since the effects of zeitgeber (which were calculated as a quotient of the motility during the light phase and the overall motility during the light and dark phase) had a value which was significantly > 0.5 (Fig. 14).

Influenced by long-term exposure of MC-LR, *Danio rerio* remained significantly diurnally active (effects of zeitgeber > 0.5), whereas *Leucaspilus delineatus* remained only diurnal active at the lowest MC-LR concentration of $0.5 \mu\text{g l}^{-1}$ (effects of zeitgeber > 0.5). At elevated concentrations of MC-LR ($5 \mu\text{g l}^{-1}$, $15 \mu\text{g l}^{-1}$ and $50 \mu\text{g l}^{-1}$) *Leucaspilus delineatus* reversed their prominent diurnal activity and the fish became significantly nocturnal (effects of zeitgeber < 0.5) (Fig. 14).

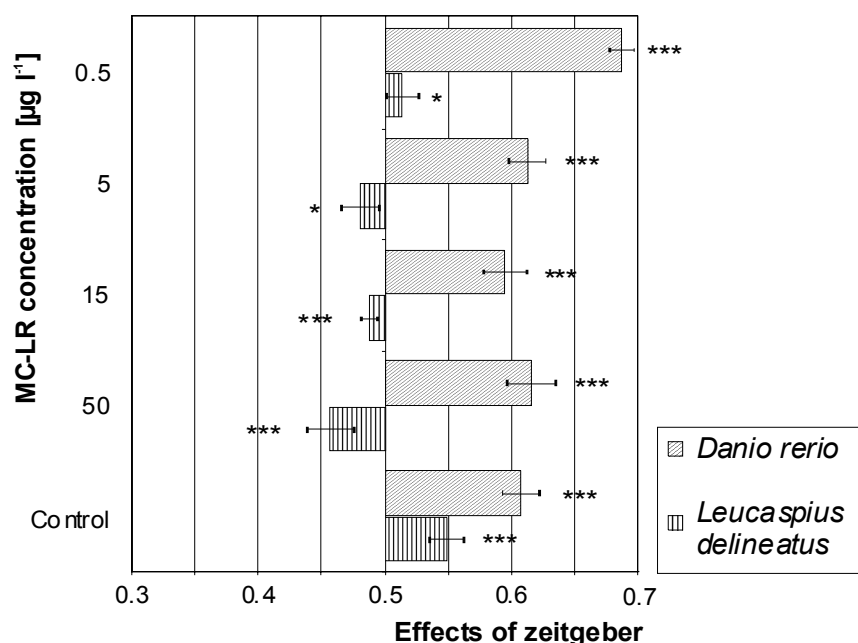


Fig. 14. Effects of zeitgeber with 95% confidence intervals for *Danio rerio* and *Leucaspilus delineatus* exposed by MC-LR. Asterisks indicate significant differences to the value of 0.5. At values between 0 and 0.5, the animals are nocturnally active and between 0.5 and 1 they are diurnally active. Means and 95% confidence intervals are shown.

5.1.7 Cosinor analysis and Polar Plots

Figures 15 and 16 show the fit of cosinor regression to the original data series. F-statistics and rhythmical parameters of cosinor analysis: MESOR, amplitude, acrophase and period length, are shown in Table 6.

Obviously the exposure to MC-LR altered the motility rhythm compared to the control. For *Danio rerio* all tested MC-LR concentrations as well as the control showed clearly single peaks of motility (Fig. 15). In contrast, for *Leucaspius delineatus* MC-LR concentrations of $5 \mu\text{g l}^{-1}$, $15 \mu\text{g l}^{-1}$ and $50 \mu\text{g l}^{-1}$ led to clear double peaks of motility, whereby the control and the lowest MC-LR concentration of $0.5 \mu\text{g l}^{-1}$ showed single peaks of motility (Fig. 16).

For *Danio rerio* the lowest MC-LR concentration of $0.5 \mu\text{g l}^{-1}$ led to a significant increase in the circadian motility amplitude by 2.96 pix s^{-1} (Tab. 6). Elevated MC-LR concentration of $5 \mu\text{g l}^{-1}$ significantly increased the MESOR by 2.23 pix s^{-1} . The highest test concentration of $50 \mu\text{g l}^{-1}$ led to a significant decrease of MESOR (by 1.83 pix s^{-1}) as well as amplitude (by 2.2 pix s^{-1}). The period of the activity cycles was stable in all cases without any significant differences and showed a pronounced circadian rhythm (Tab. 6).

For *Leucaspius delineatus* an MC-LR concentration of $0.5 \mu\text{g l}^{-1}$ significantly increased the MESOR by 2.98 pix s^{-1} (Tab. 4). Elevated concentrations of $5 \mu\text{g l}^{-1}$ and $15 \mu\text{g l}^{-1}$ significantly increased the MESOR by 2.68 pix s^{-1} and 1.69 pix s^{-1} , respectively. At the highest MC-LR concentration of $50 \mu\text{g l}^{-1}$, the MESOR was significantly smaller than that in the controls by 2.03 pix s^{-1} . At this concentration of $50 \mu\text{g l}^{-1}$ the amplitude tended to decrease, but this was not statistically relevant. The circadian period of the activity cycles was stable only at the lowest test concentration of $0.5 \mu\text{g l}^{-1}$. All elevated MC-LR concentrations of $5 \mu\text{g l}^{-1}$, $15 \mu\text{g l}^{-1}$ and $50 \mu\text{g l}^{-1}$ reduced the period length significantly and clearly revealed an ultradian rhythm with a cycle length $< 20\text{h}$ (Tab. 6).

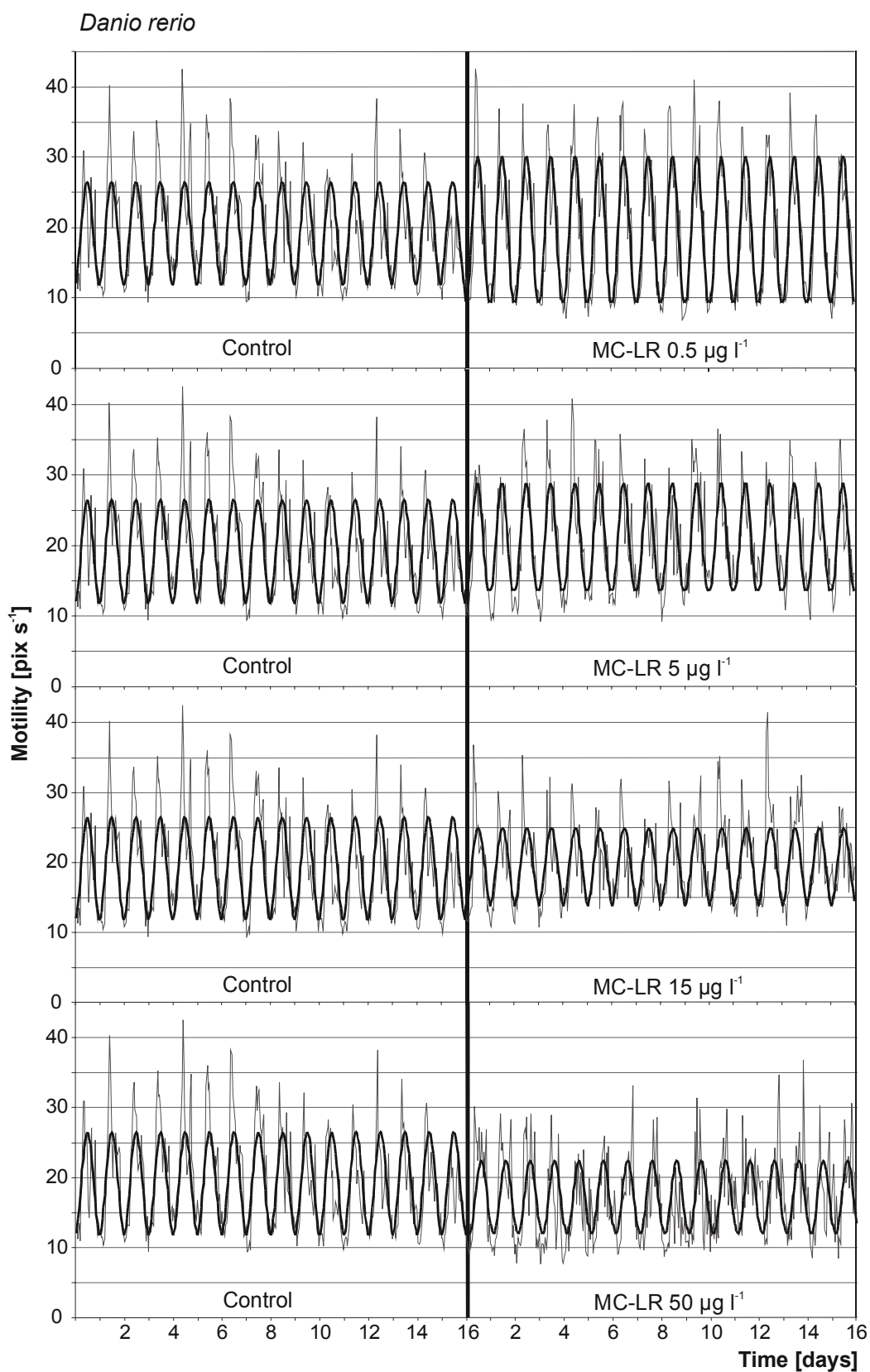


Fig. 15. Circadian motility rhythms of *Danio rerio* determined by the cosinor method. Comparison of control and MC-LR exposed groups. Average motilities per hour (grey line) and cosinor fitted curves (black line).

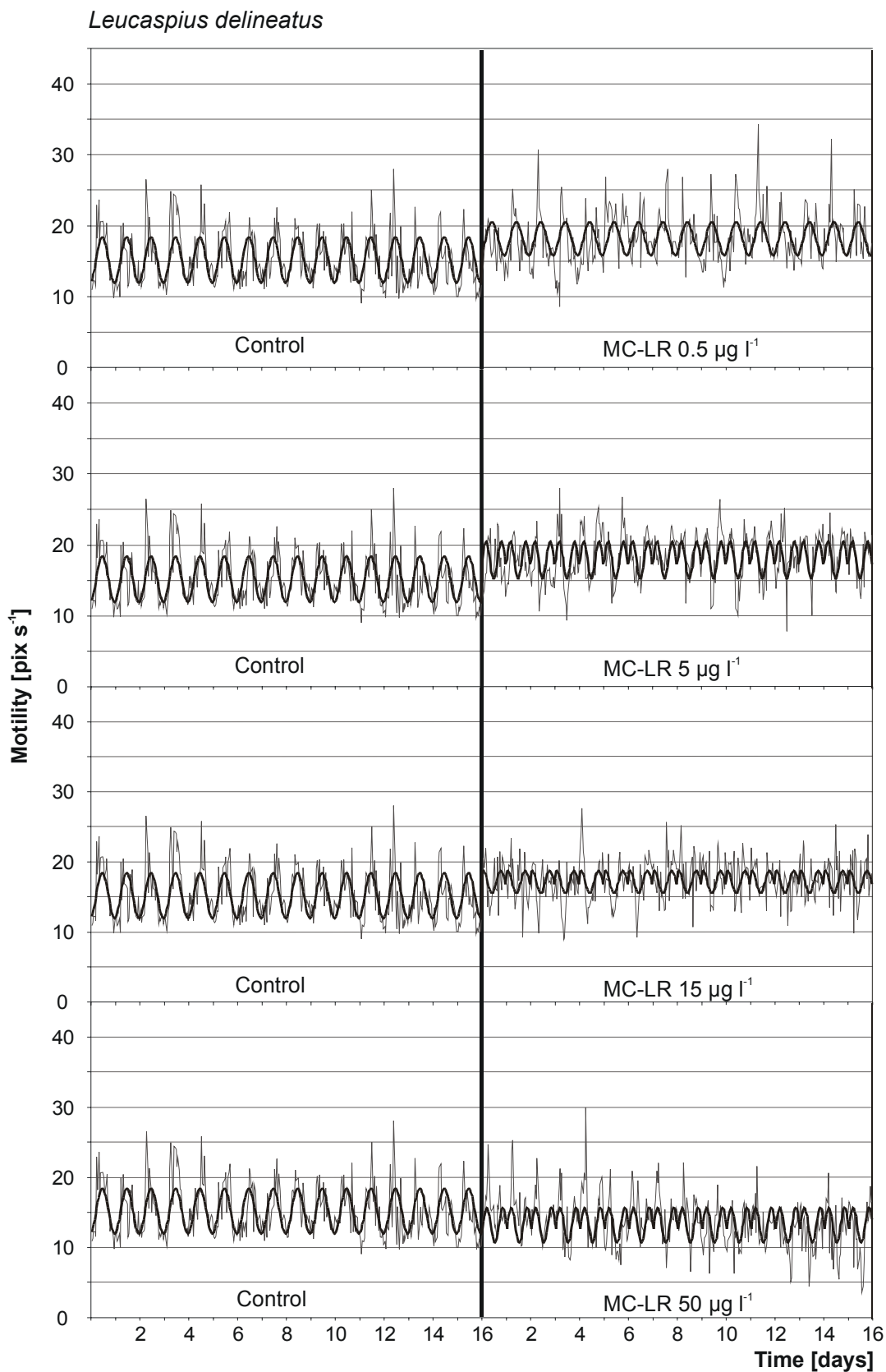


Fig. 16. Circadian motility rhythms of *Leucaspius delineatus* determined by the cosinor method. Comparison of control and MC-LR exposed groups. Average motilities per hour (grey line) and cosinor fitted curves (black line).

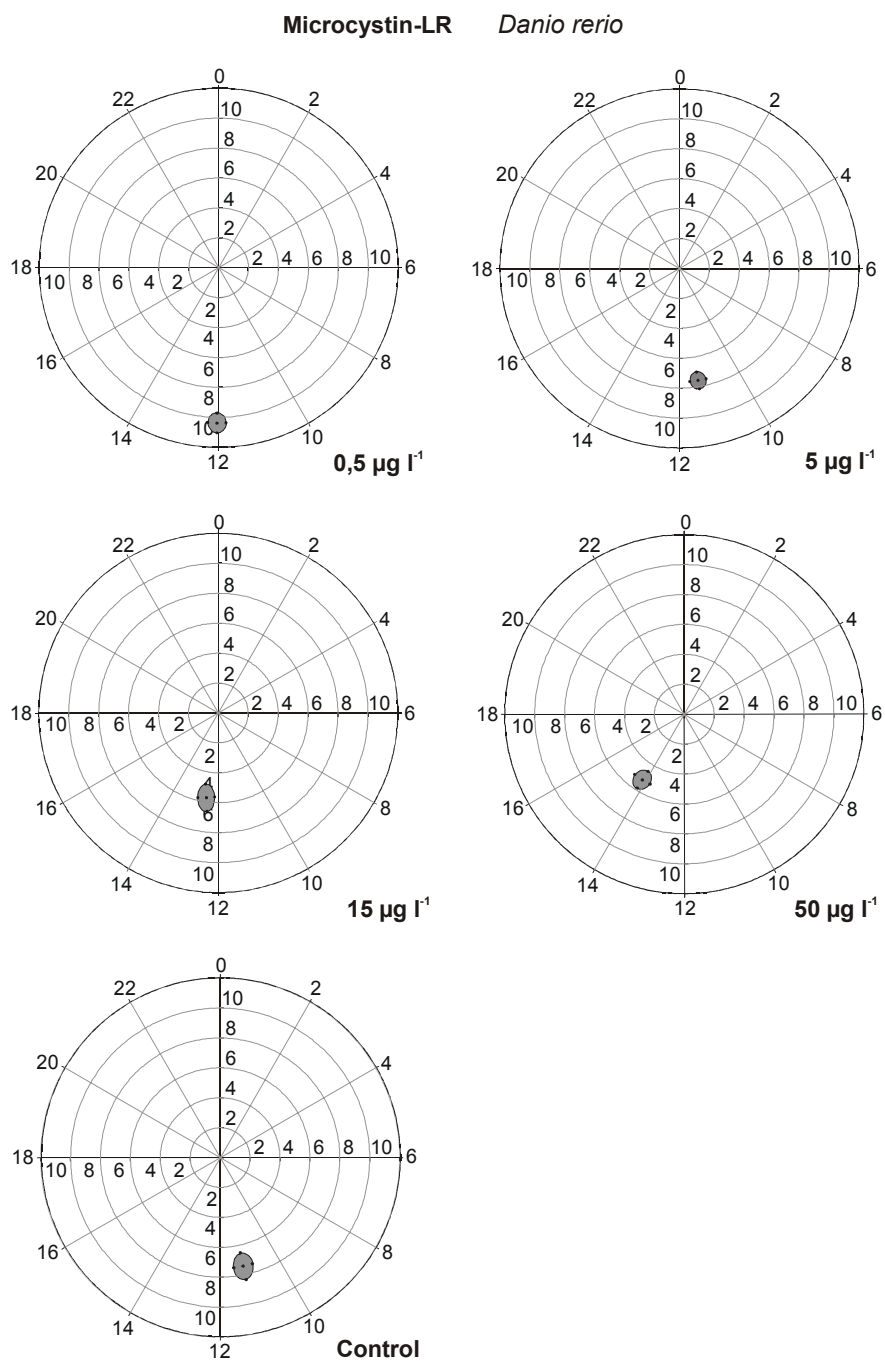


Fig. 17. Polarogram of cosinor analysis for *Danio rerio* exposed by MC-LR. Angular axis represents the amplitude [pix s⁻¹]. Radial axis represents the time of the day [h]. Confidence ellipse covers the 95% interval.

The rhythmical parameters amplitude and acrophase are graphically represented in the polar plots (Fig. 17 and Fig. 18).

For *Danio rerio* the acrophase delayed significantly at MC-LR concentrations of 0.5 µg l⁻¹, 15 µg l⁻¹ and 50 µg l⁻¹ compared to the control group. At an MC-LR

concentration of $0.5 \mu\text{g l}^{-1}$ the acrophase delayed for 51 min, at a concentration of $15 \mu\text{g l}^{-1}$ it delayed for 58 min and at a concentration of $50 \mu\text{g l}^{-1}$ the acrophase delayed for 2 h and 58 min.

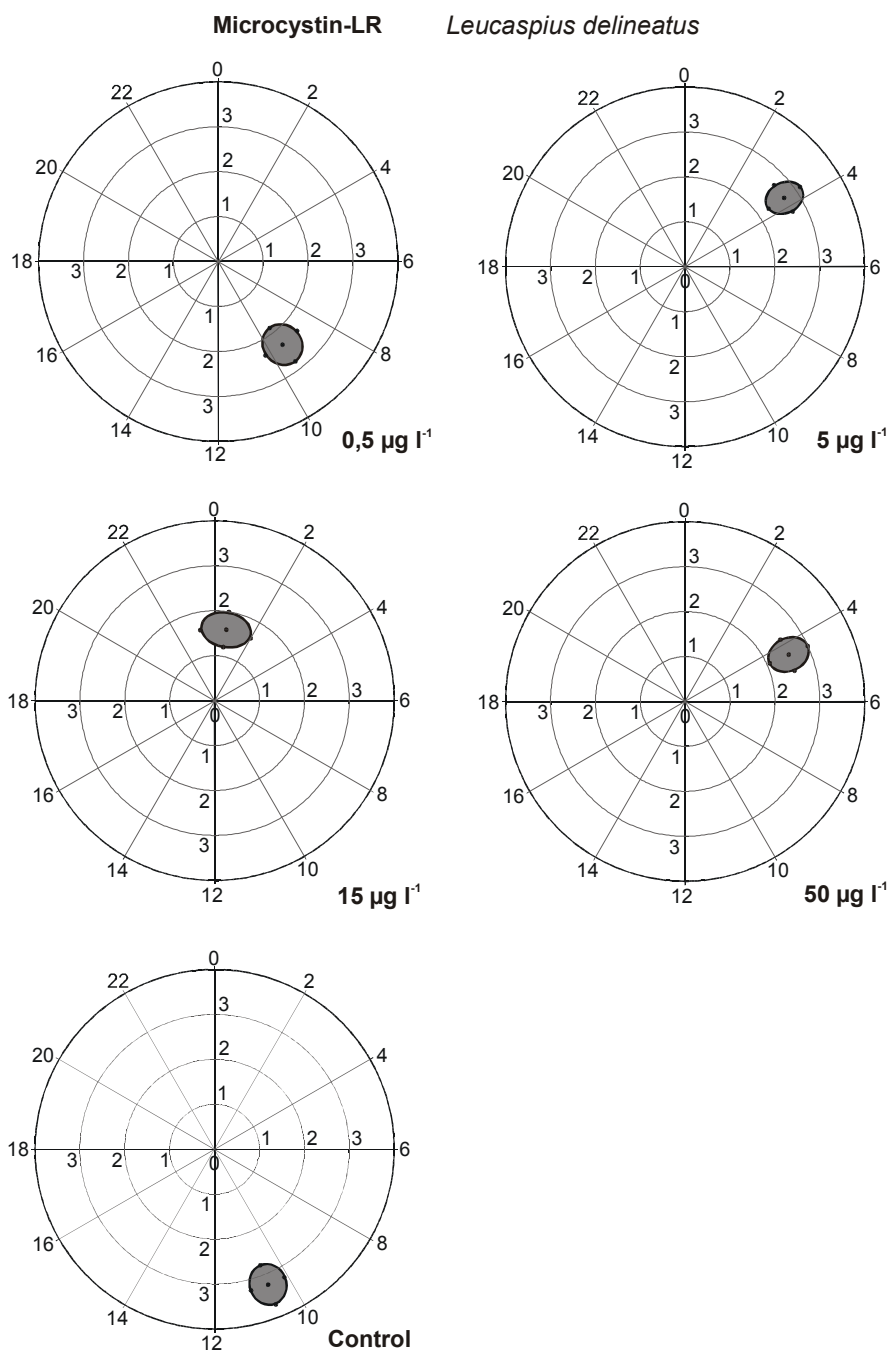


Fig. 18. Polarogram of cosinor analysis for *Leucaspis delineatus* exposed by MC-LR. Angular axis represents the amplitude [pix s^{-1}]. Radial axis represents the time of the day [h]. Confidence ellipse covers the 95% interval.

For *Leucaspis delineatus* in contrast, the acrophase advanced significantly at MC-LR concentrations of 5 $\mu\text{g l}^{-1}$, 15 $\mu\text{g l}^{-1}$ and 50 $\mu\text{g l}^{-1}$. For the MC-LR concentration of 5 $\mu\text{g l}^{-1}$ the acrophase advanced for 6 h and 53 min, for the concentration of 15 $\mu\text{g l}^{-1}$ it advanced for 9 h and 57 min and for the concentration of 50 $\mu\text{g l}^{-1}$ the acrophase advanced for 6 h and 12 min.

Tab. 6. Rhythmical parameters of cosinor analysis: MESOR (M), amplitude (A), acrophase (K) and period length (P) for *Danio rerio* and *Leucaspis delineatus* exposed by microcystin-LR (* > means that the value of the exposure group is significantly higher and * < means that the value of the exposure group is significantly lower compared to the control at the 0.05% level; n.s. means that there are no significant differences between the exposure group and the control group). The F- and p-values indicate the overall difference between the exposure group and the control.

Species	Parameter	MC-LR concentration ($\mu\text{g l}^{-1}$)					Contr.			
		0.5	5	15	50					
<i>Danio rerio</i>	F-value	2087.2	2739.7	2729.7	1441.6	2032.2				
	p-value	0.000	0.000	0.000	0.000	0.000				
	M (pix s^{-1})	19.73	n.s.	21.27	* >	19.25	n.s.	17.21	* <	19.04
	A (pix s^{-1})	10.38	* >	7.59	n.s.	5.67	n.s.	5.22	* <	7.42
	K (hours)	12:03	* >	11:22	n.s.	12:10	* >	14:10	* >	11.12
	P (hours)	22:55	n.s.	22:03	n.s.	26:21	n.s.	23:21	n.s.	25.14
<i>Leucaspis delineatus</i>	F-value	3385.5	4188.63	3908.6	1912.8	2929.1				
	p-value	0.000	0.000	0.000	0.000	0.000				
	M (pix s^{-1})	18.14	* >	17.84	* >	17.12	* >	13.13	* <	15.16
	A (pix s^{-1})	2.34	n.s.	2.69	n.s.	1.60	* <	2.53	n.s.	3.24
	K (hours)	9:29	n.s.	3:41	* <	0:37	* <	4:22	* <	10.34
	P (hours)	24:55	n.s.	15:00	* <	17:29	* <	15:34	* <	24.23

5.1.8 Periodic frequency analysis

Like the cosinor analysis the power spectral analysis (Fig. 19) revealed that there is a significant circadian component (of 24 hours) which determined the swim-

ming activity of *Danio rerio* and *Leucaspius delineatus* under unexposed conditions.

For *Danio rerio* the periods of 24 hours, 12 hours, 8 hours and 6 hours explained a significant proportion of the total variation under unexposed conditions. For all exposed groups of *Danio rerio*, the significance of these rhythms remained, whereby there was additionally a significant period of 4.8 hours compared to the control. For *Danio rerio* the explained proportion of the 24 hours period increased at lower MC-LR concentrations of 0.5 and 5 $\mu\text{g l}^{-1}$ and decreased at elevated MC-LR concentrations of 15 and 50 $\mu\text{g l}^{-1}$ (Fig. 19). The power spectrum of MC-LR concentrations of 15 $\mu\text{g l}^{-1}$ is equal to that of the control for *Danio rerio*.

For *Leucaspius delineatus* the periods of 24 hours, 12 hours, 8 hours, 6 hours and 4.8 hours explained a significant proportion of the total variation under unexposed conditions. The significance of the rhythm components of 24 hours and 12 hours remained for all exposed groups. However the amplitude of circadian rhythm decreased under exposure and furthermore at elevated MC-LR concentrations of 5 $\mu\text{g l}^{-1}$, 15 $\mu\text{g l}^{-1}$ and 50 $\mu\text{g l}^{-1}$ the dominance of circadian rhythms ($\tau = 24 \text{ h}$) was clearly reduced and simultaneously the proportion of a harmonic oscillation with a 12 hours rhythm increased.

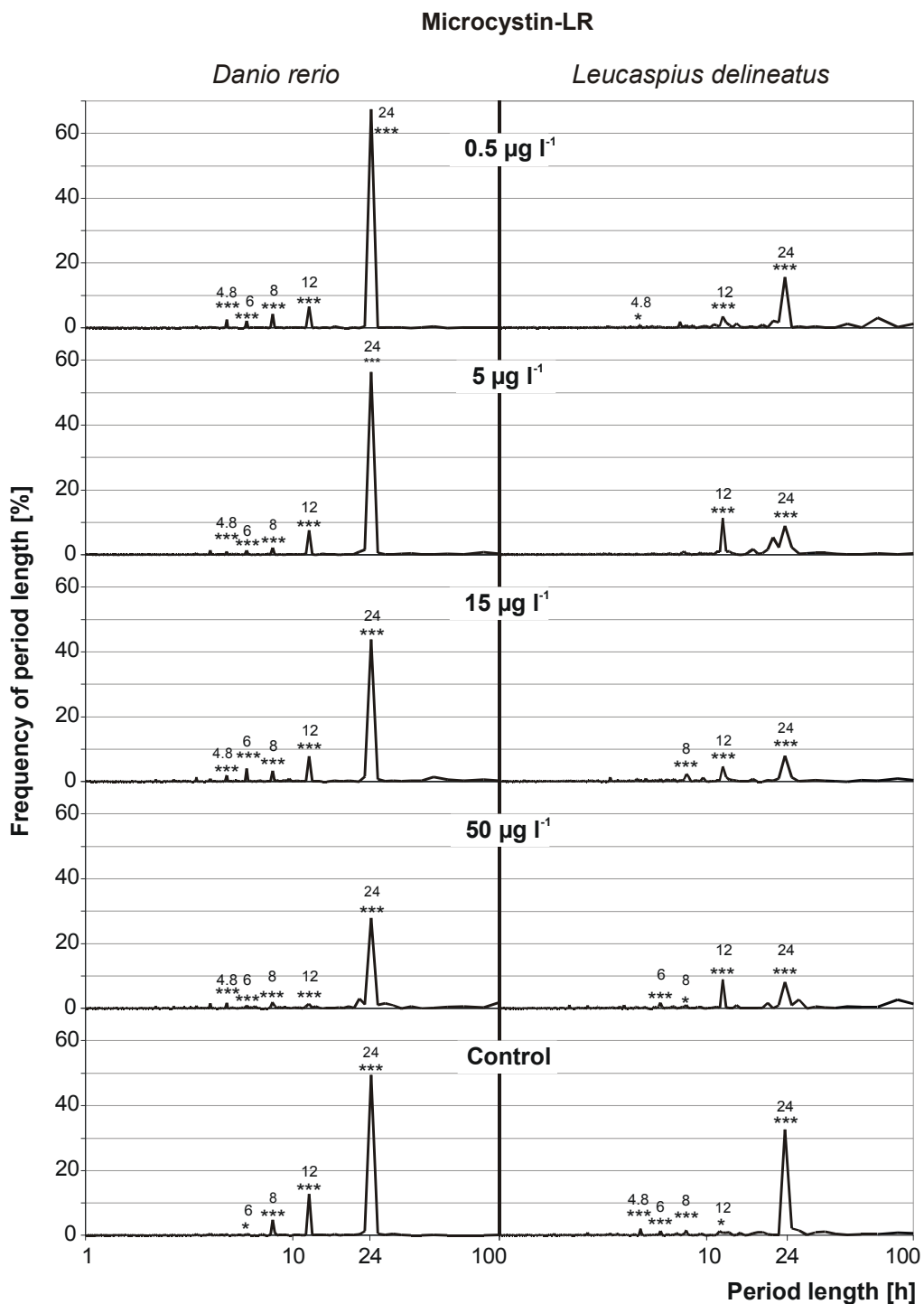


Fig. 19. Power spectrum of motility of *Danio rerio* and *Leucaspis delineatus* exposed to MC-LR.

For the MC-LR experiment with *Danio rerio* the DFC (Degree of Functional Coupling) was 90.4% and the harmonic portion was 68.4% for the controls (Tab. 7). As the explained proportion of the 24 hours period the DFC and the harmonic portion increased at lower MC-LR concentrations of 0.5 and 5 $\mu\text{g l}^{-1}$ and decreased at elevated MC-LR concentrations of 15 and 50 $\mu\text{g l}^{-1}$.

For *Leucaspilus delineatus* the DFC was 76.8% and the harmonic portion was 39.2% for the control. In all MC-LR exposed groups both DFC value and harmonic portion were decreased (Tab. 7).

Tab. 7. Degree of Functional Coupling (DFC) and the harmonic portion (HP) of *Danio rerio* and *Leucaspilus delineatus* exposed by MC-LR.

Species	Parameter	MC- LR concentration ($\mu\text{g l}^{-1}$)				Control
		0.5	5	15	50	
<i>Danio rerio</i>	DFC [%]	98.37	93.54	89.43	67.52	90.44
	HP [%]	85.25	70.54	64.57	37.82	68.40
<i>Leucaspilus</i>	DFC [%]	49.53	52.93	69.15	59.26	76.78
<i>delineatus</i>	HP [%]	20.16	20.24	16.31	23.05	39.25

5.2 Effects of Trichlorobiphenyl

5.2.1 Mean motility over the whole exposure period

The mean motility of *Danio rerio* was significantly decreased when exposed to PCB 28 at concentrations of 100 $\mu\text{g l}^{-1}$ and 150 $\mu\text{g l}^{-1}$ (Fig. 20).

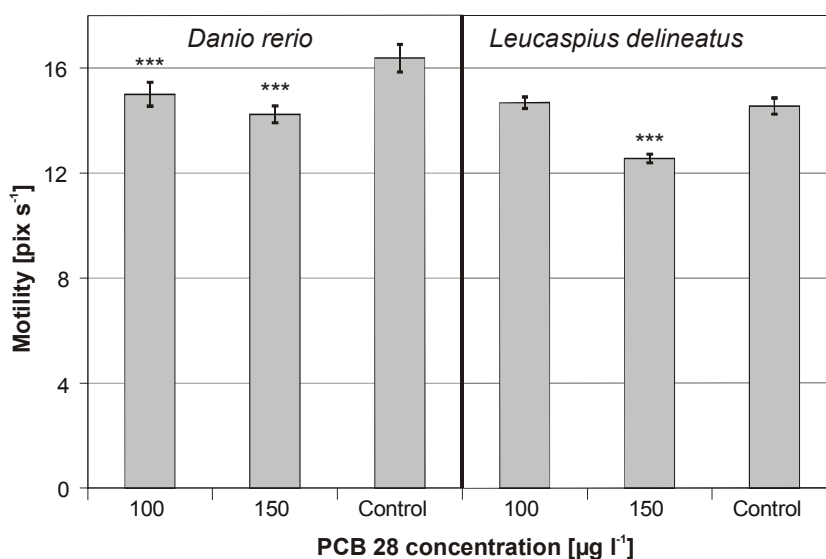


Fig. 20. Average motility of *Danio rerio* and *Leucaspis delineatus* exposed by PCB 28 over the whole measuring time per day (23 h per day) and the whole exposure period. Means and 95% confidence are shown.

For *Leucaspis delineatus* the highest test concentration of 150 $\mu\text{g l}^{-1}$ led to a significant decrease of motility, too, whereas the lower PCB 28 concentration of 100 $\mu\text{g l}^{-1}$ did not change the motility significantly (Fig. 20). Comparing the effects of PCB 28 between the duplicates of both fish species there were no significant differences at the 0.05 level between each duplicates of 100 $\mu\text{g l}^{-1}$ and 150 $\mu\text{g l}^{-1}$ and between the control groups.

5.2.2 Mean motility during light and dark phases

With *Danio rerio* the lower PCB 28 exposure concentration of 100 $\mu\text{g l}^{-1}$ led to a significant decrease in motility during the light phase and dark phase over the whole exposure period (Fig. 21). The elevated PCB 28 exposure of 150 $\mu\text{g l}^{-1}$ led to a significantly decreased motility during the light phase. As it is shown in the daily activity curves (Fig. 23) the reaction of *Danio rerio* to the onset of light in-

indicated the influence of PCB 28 very clearly. For three hours after the onset of light the motility of *Danio rerio* was significantly reduced under the influence of PCB 28 for both test concentrations of $100 \mu\text{g l}^{-1}$ and $150 \mu\text{g l}^{-1}$ (Fig. 22).

With *Leucaspilus delineatus* a PCB 28 concentration of $100 \mu\text{g l}^{-1}$ caused a significant decrease in motility during the light phase, whereas the motility was significantly increased during the dark phase. At the concentration of $150 \mu\text{g l}^{-1}$ the motility of the light as well as dark phase was significantly decreased (Fig. 22). As it was shown for *Danio rerio* the reaction of *Leucaspilus delineatus* to the onset of light was significantly reduced under PCB 28 exposure (Fig. 22, Fig. 23).

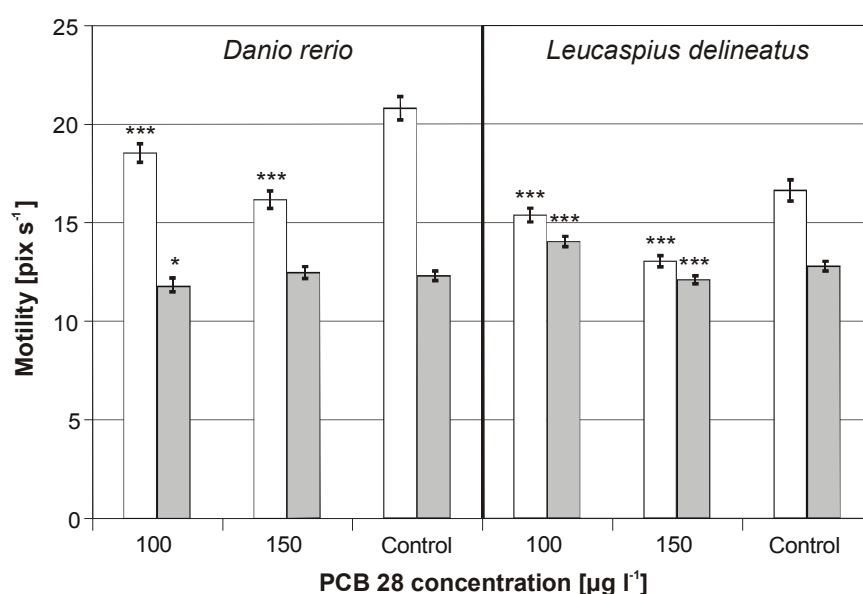


Fig. 21. Average motility of *Danio rerio* and *Leucaspilus delineatus* divided in light (white bars) and dark phases (grey bars) over the whole period of exposure to PCB 28. Means and 95% confidence intervals are shown.

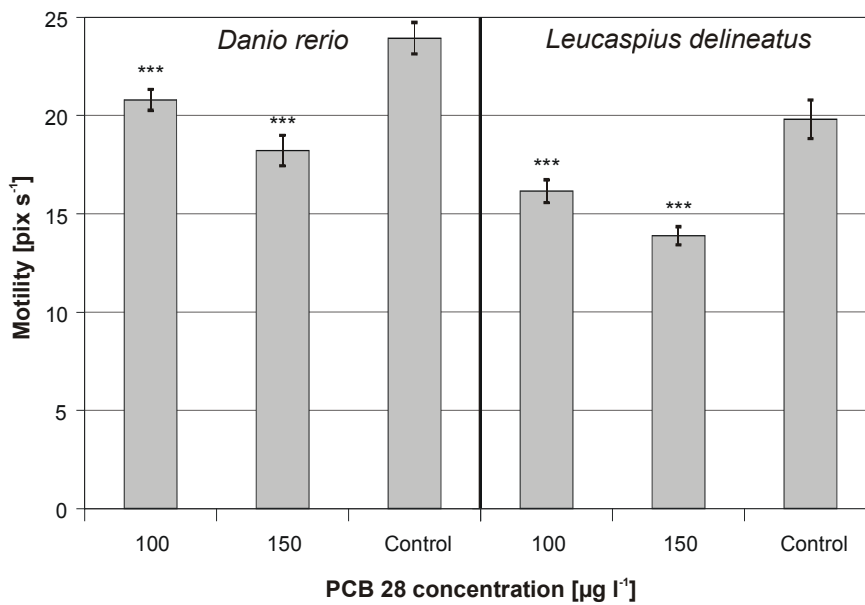


Fig. 22. Average motility of *Danio rerio* and *Leucaspilus delineatus* during the time interval of 3 hours after the onset of light over the whole period of exposure to PCB 28. Means and 95% confidence intervals are shown.

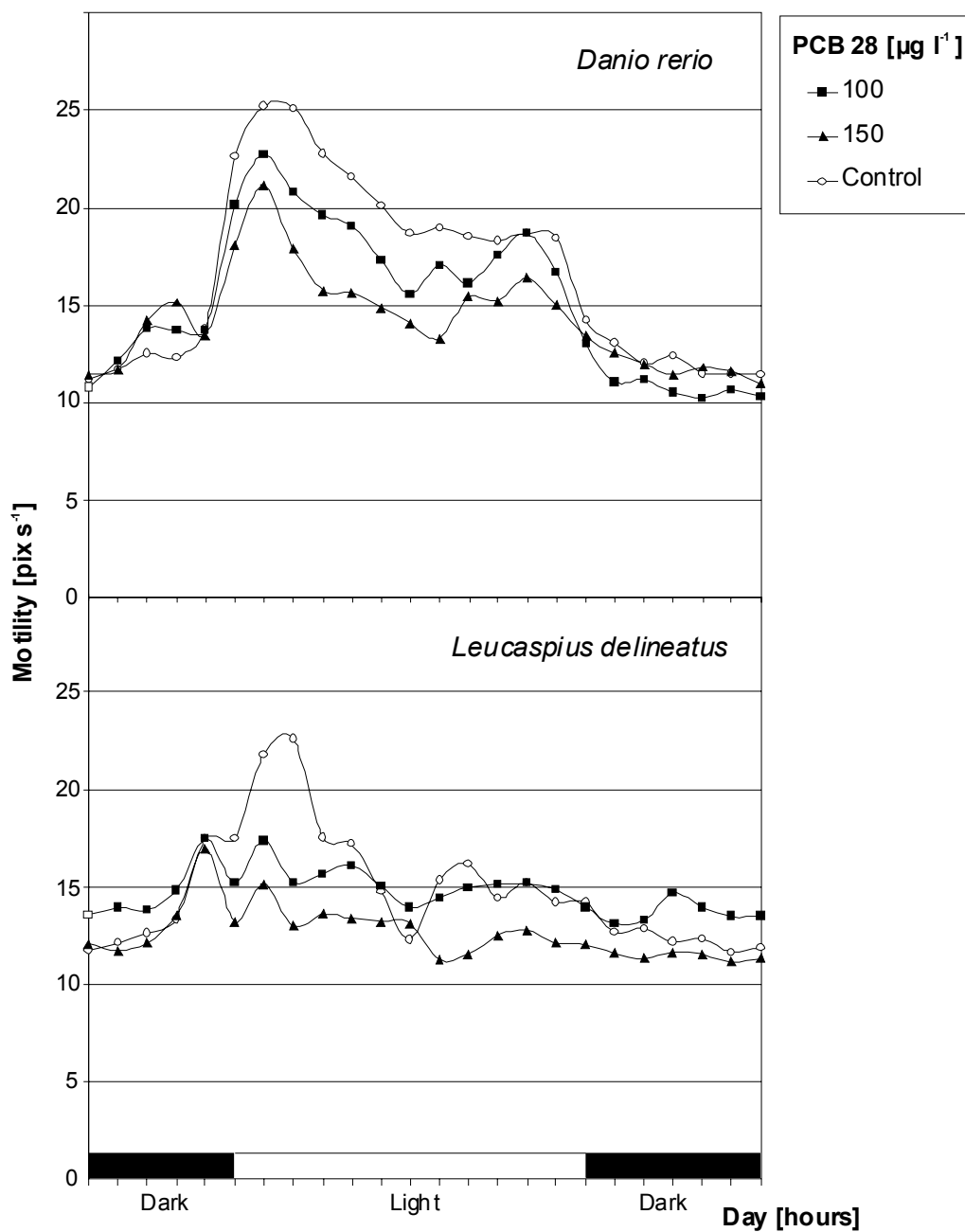


Fig. 23. Smoothed curve of average motility of *Danio rerio* and *Leucaspilus delineatus* over the whole period of exposure to PCB 28.

5.2.3 Temporal development of mean motilities

For analysing the temporal development of mean motilities the whole period of exposure to PCB 28 was divided into four time intervals of two days each. The mean motility of every exposure group was calculated for each of the four intervals, distinguished between daylight and nighttime activity.

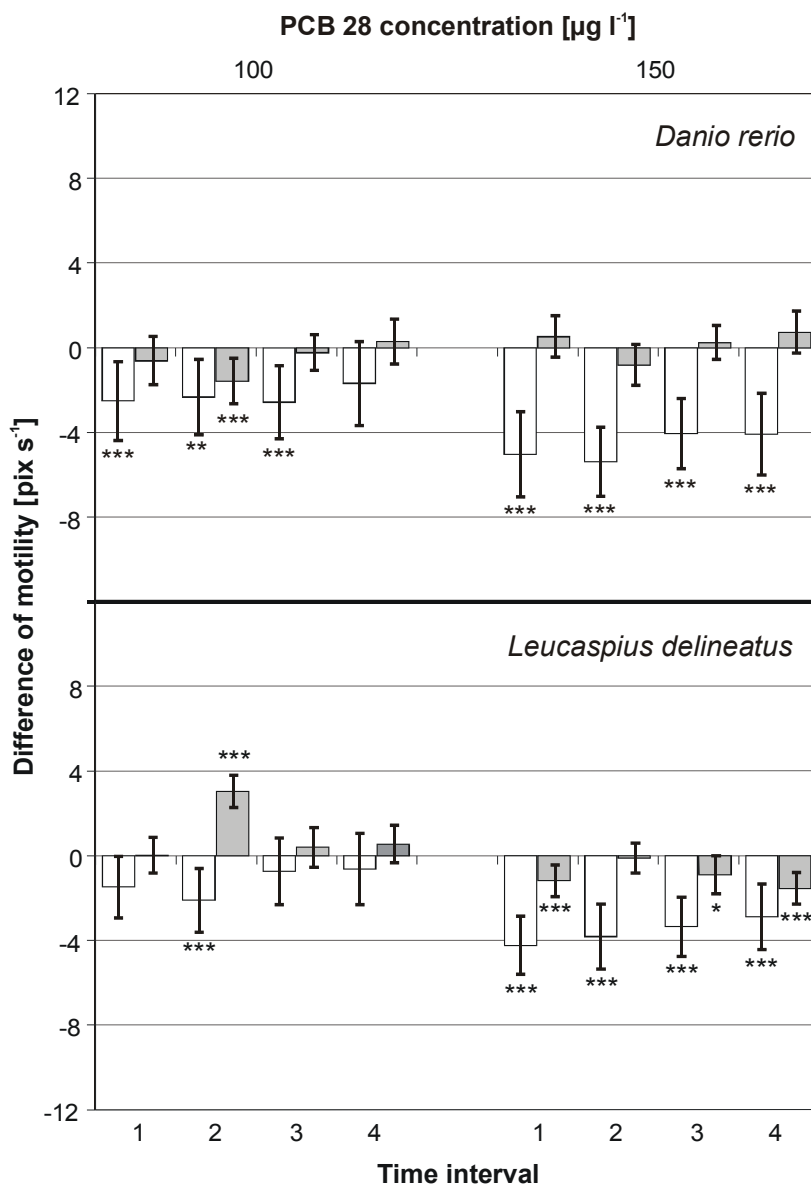


Fig. 24. Differences in motility between the different exposure groups and the controls for *Danio rerio* and *Leucaspis delineatus*, divided into light (white bars) and dark phases (grey bars). The whole period of exposure to PCB 28 was divided into four time intervals of 2 days each. Means and 95% confidence intervals are shown.

With *Danio rerio* during light phases, the decrease in motility caused by the PCB 28 concentration of $100 \mu\text{g l}^{-1}$ occurred from the first to the third exposure interval and disappeared in the last exposure interval. During dark phases, the motility at the exposure concentration of $100 \mu\text{g l}^{-1}$ decreased significantly only in the second exposure interval. During the light phase a PCB 28 concentration of $150 \mu\text{g l}^{-1}$ caused a decrease in motility from the first exposure interval which continued up to the last exposure interval, whereas during the dark phase there were no significant differences in any exposure period (Fig. 24).

During the light phase, the motility of *Leucaspilus delineatus* at the concentration of $100 \mu\text{g l}^{-1}$ significantly decreased only in the second exposure interval and only for this second interval the motility during the dark phase was significantly increased. The PCB 28 exposure concentration of $150 \mu\text{g l}^{-1}$ led to a significant decrease of daytime motility in all four exposure intervals, whereas the motility during the night was significantly decreased in the first, third and last exposure interval (Fig. 24).

5.2.4 Number of turns over the whole exposure period

With *Danio rerio*, the number of turns was significantly decreased at the PCB 28 concentrations of $100 \mu\text{g l}^{-1}$ and of $150 \mu\text{g l}^{-1}$ during the light phases (Fig. 25). Only at the higher PCB 28 concentration of $150 \mu\text{g l}^{-1}$ the number of turns was significantly increased during the dark phase.

With *Leucaspilus delineatus*, the number of turns did not change significantly at the lower PCB 28 concentration of $100 \mu\text{g l}^{-1}$ (Fig. 25). In contrast, the higher exposure concentration of $150 \mu\text{g l}^{-1}$ caused a significant decrease of the number of turns during both the light and dark phases.

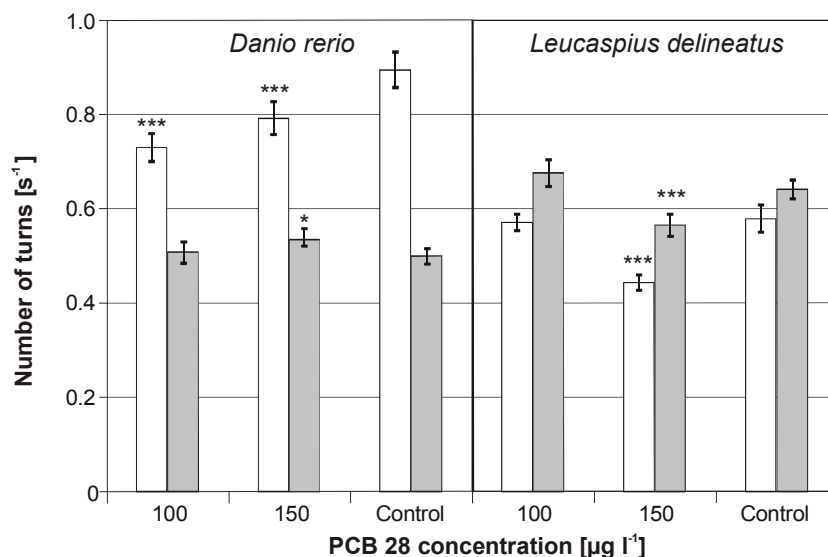


Fig. 25. Average number of turns of *Danio rerio* and *Leucaspilus delineatus* divided in light (white bars) and dark phases (grey bars) over the whole period of exposure to PCB 28. Means and 95% confidence intervals are shown.

5.2.5 Regression between motilities and turns

For analysing the swimming mode a regression between motility versus number of turns was fitted. With increasing motility the number of turns increased in all controls and PCB 28 exposed test groups of both species, described by a linear function (Tab. 8, Fig. 26). For *Danio rerio* under unexposed conditions, the number of turns increased with increasing motility with an determination coefficient of $r^2 = 0.939$. The determination coefficient, the slope and the intercept did not change significantly under exposure of PCB 28 for *Danio rerio*. This indicated a similar statistic relation between the increasing number of turns and increasing motility of the exposed groups compared with the control.

For *Leucaspilus delineatus* under unexposed conditions, with increasing motility, number of turns increased with an determination coefficient of 0.382 (Tab. 8, Fig. 26). In contrast to *Danio rerio* elevated concentrations of PCB 28 led to an increase of the overall determination coefficient compared to the control for *Leucaspilus delineatus* indicating that the statistic relation between increase of number of turns and increasing motility was stronger under exposure. The slopes of the regressions were significantly increased under PCB 28 exposure.

Tab. 8. Number of turns over motility with fitted linear function: $y = a \cdot x + b$ for *Danio rerio* and *Leucaspilus delineatus* exposed by PCB 28.

Species	Parameter	PCB 28 concentration ($\mu\text{g l}^{-1}$)		
		100	150	Control
<i>Danio rerio</i>	a (slope)	0.052	0.051	0.053
	p-value	***	***	***
	b (intercept)	-0.144	-0.106	-0.142
	p-value	***	***	***
	r^2	0.951	0.845	0.939
	F-value	3513.222	995.004	2801.652
	p-value	***	***	***
<i>Leucaspilus delineatus</i>	a (slope)	0.040	0.050	0.022
	p-value	***	***	***
	b (intercept)	0.033	-0.121	0.283
	p-value		*	***
	r^2	0.424	0.500	0.382
	F-value	134,020	182,020	112,405
	p-value	***	***	***

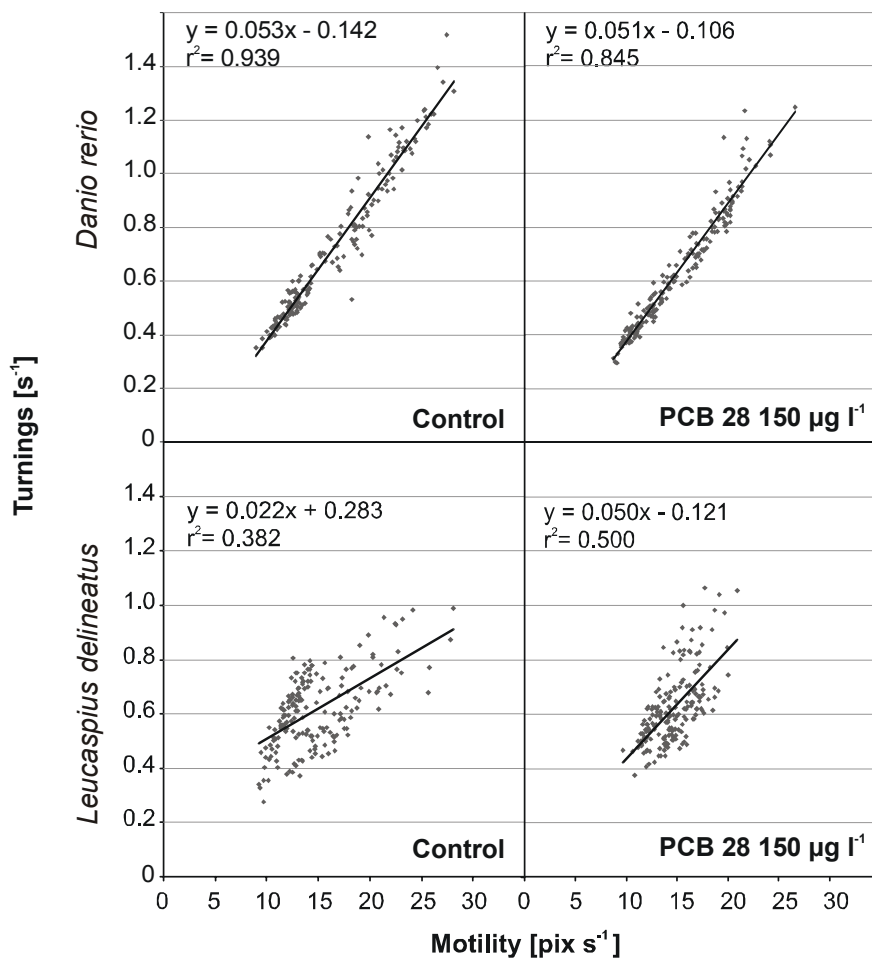


Fig. 26. Number of turns over motility with fitted linear function: $y = a \cdot x + b$ for *Danio rerio* and *Leucaspis delineatus* exposed by PCB 28.

5.2.6 Effects of zeitgeber

Both *Danio rerio* and *Leucaspilus delineatus* revealed a significant diurnal activity under unexposed conditions since the effects of zeitgeber (which were calculated as a quotient of the motility during the light phase and the overall motility during the light and dark phase) had a value which is clearly higher than 0.5 (Fig. 27). Influenced by PCB 28 *Danio rerio* and *Leucaspilus delineatus* remained significantly diurnal active. For *Danio rerio* and *Leucaspilus delineatus* the values of the effects of zeitgeber were significantly reduced for the exposure groups (100 and 150 $\mu\text{g l}^{-1}$) compared to the control groups.

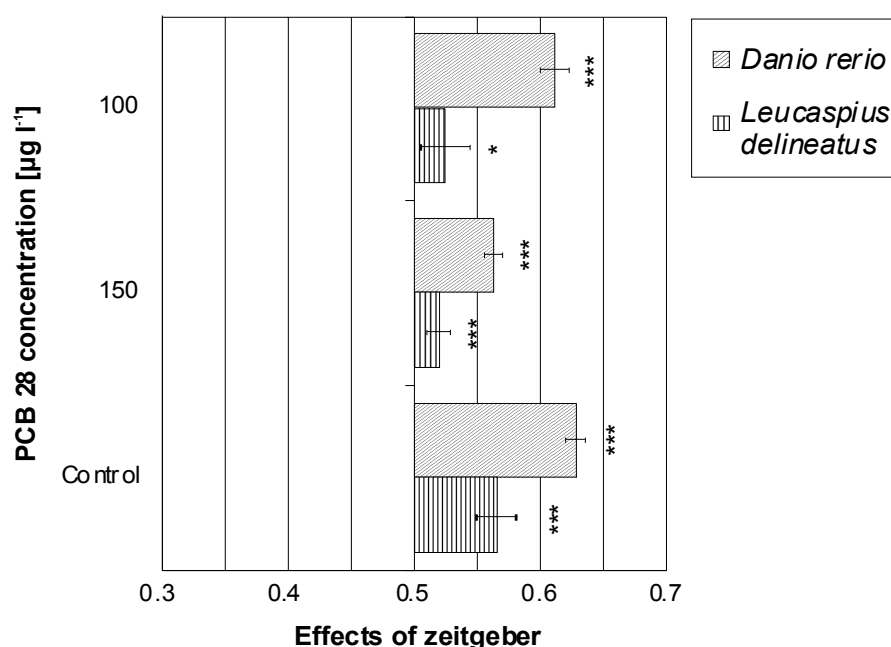


Fig. 27. Effects of zeitgeber with 95% confidence intervals for *Danio rerio* and *Leucaspilus delineatus* exposed by PCB 28. Asterisks indicate significant differences to the value of 0.5. At values between 0 and 0.5, the animals are nocturnal active and between 0.5 and 1 they are diurnal active. Means and 95% confidence intervals are shown.

5.2.7 Cosinor analysis and Polar Plots

The rhythmical parameters of cosinor analysis: MESOR, amplitude, acrophase and period length, are shown in Table 9. Figure 28 and Figure 29 show the fit of cosinor regression to the original data series of the PCB 28 test. The rhythmical parameter amplitude and acrophase are graphically represented in the polar plots (Fig. 30).

The exposure to PCB 28 altered the expression of the motility rhythm of both fish species compared with the control values.

Tab. 9. Rhythmical parameters of cosinor analysis: MESOR (M), amplitude (A), acrophase (K) and period length (P) for *Danio rerio* and *Leucaspius delineatus* exposed by PCB 28. (* > means that the value of the exposure group is significantly higher and * < means that the value of the exposure group is significantly lower compared to the control at the 0.05 % level; n.s. means that there are no significant differences between the exposure group and the control group). The F- and p-values indicate the overall difference between the exposure group and the control.

Species	Parameter	PCB 28 concentration ($\mu\text{g l}^{-1}$)			
		100		150	Contr.
<i>Danio rerio</i>	F-value	2822.4		3098.6	2662.1
	p-value	0.000		0.000	0.000
	M (pix s^{-1})	14.79	* <	13.88	* < 16.71
	A (pix s^{-1})	4.75	* <	2.71	* < 5.68
	K (hours)	9:10	n.s.	8:38	n.s. 9:27
	P (hours)	22:55	n.s.	28:19	n.s. 23:05
<i>Leucaspius delineatus</i>	F-value	2578.8		3673.37	1470.1
	p-value	0.000		0.000	0.000
	M (pix s^{-1})	14.72	n.s.	12.59	* < 14.87
	A (pix s^{-1})	1.15	* <	1.34	* < 3.13
	K (hours)	8:09	n.s.	6:52	* < 8:28
	P (hours)	23:33	n.s.	23:58	n.s. 22:46

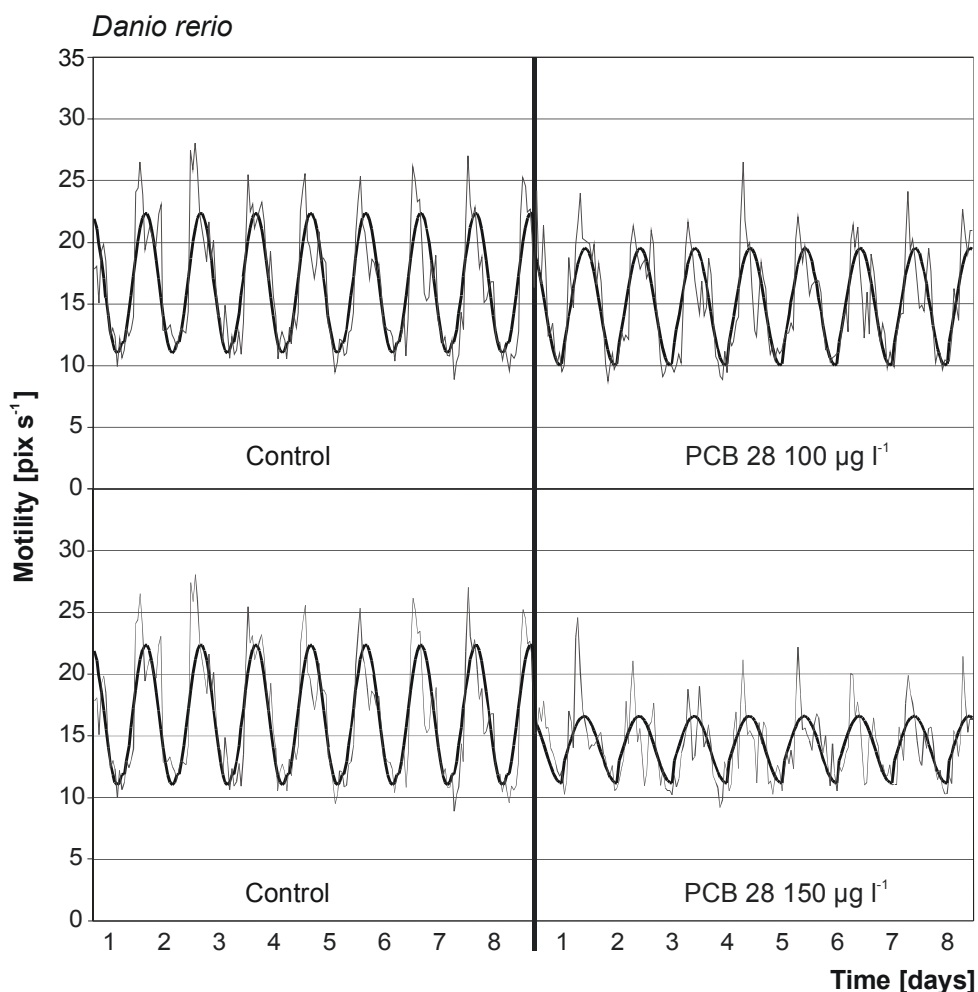


Fig. 28. Circadian motility rhythms of *Danio rerio* determined by the cosinor method. Comparison of control and PCB 28 exposed groups. Average motilities per hour (grey line) and cosinor fitted curves (black line).

For *Danio rerio* a PCB 28 concentration of $100 \mu\text{g l}^{-1}$ led to a significant decrease in the MESOR by 1.93 pix s^{-1} as well as the circadian motility amplitude by 0.93 pix s^{-1} (Tab. 9). The elevated PCB 28 concentration of $150 \mu\text{g l}^{-1}$ led to a significant decrease of MESOR by 2.83 pix s^{-1} and amplitude by 2.97 pix s^{-1} . The period of the activity cycles was stable in all cases without any significant differences and showed a pronounced circadian rhythm. Furthermore the rhythmical parameter acrophase did not change significantly influenced by PCB 28.

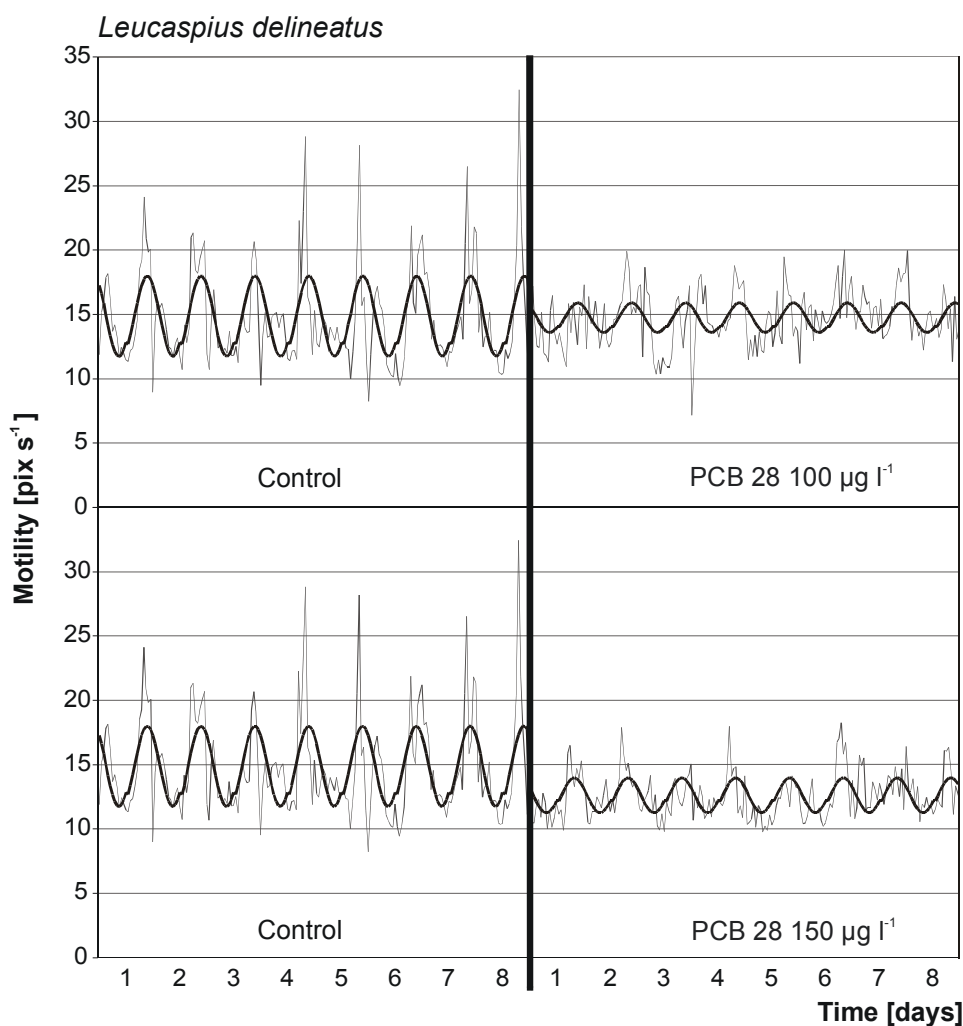


Fig. 29. Circadian motility rhythms of *Leucaspilus delineatus* determined by the cosinor method. Comparison of control and PCB 28 exposed groups. Average motilities per hour (grey line) and cosinor fitted curves (black line).

For *Leucaspilus delineatus* a PCB 28 concentrations of $100 \mu\text{g l}^{-1}$ significantly decreased the amplitude by 1.98 pix s^{-1} whereas MESOR, acrophase and period length did not change significantly. A PCB 28 concentration of $150 \mu\text{g l}^{-1}$ significantly decreased both MESOR (by 2.28 pix s^{-1}) and amplitude (by 1.79 pix s^{-1}). The acrophase advanced significantly for 1 h and 36 min at the concentration of $150 \mu\text{g l}^{-1}$. Furthermore at this concentration the period length tended to increase with a difference to the control group of 1 h and 12 min, but this change was not significant.

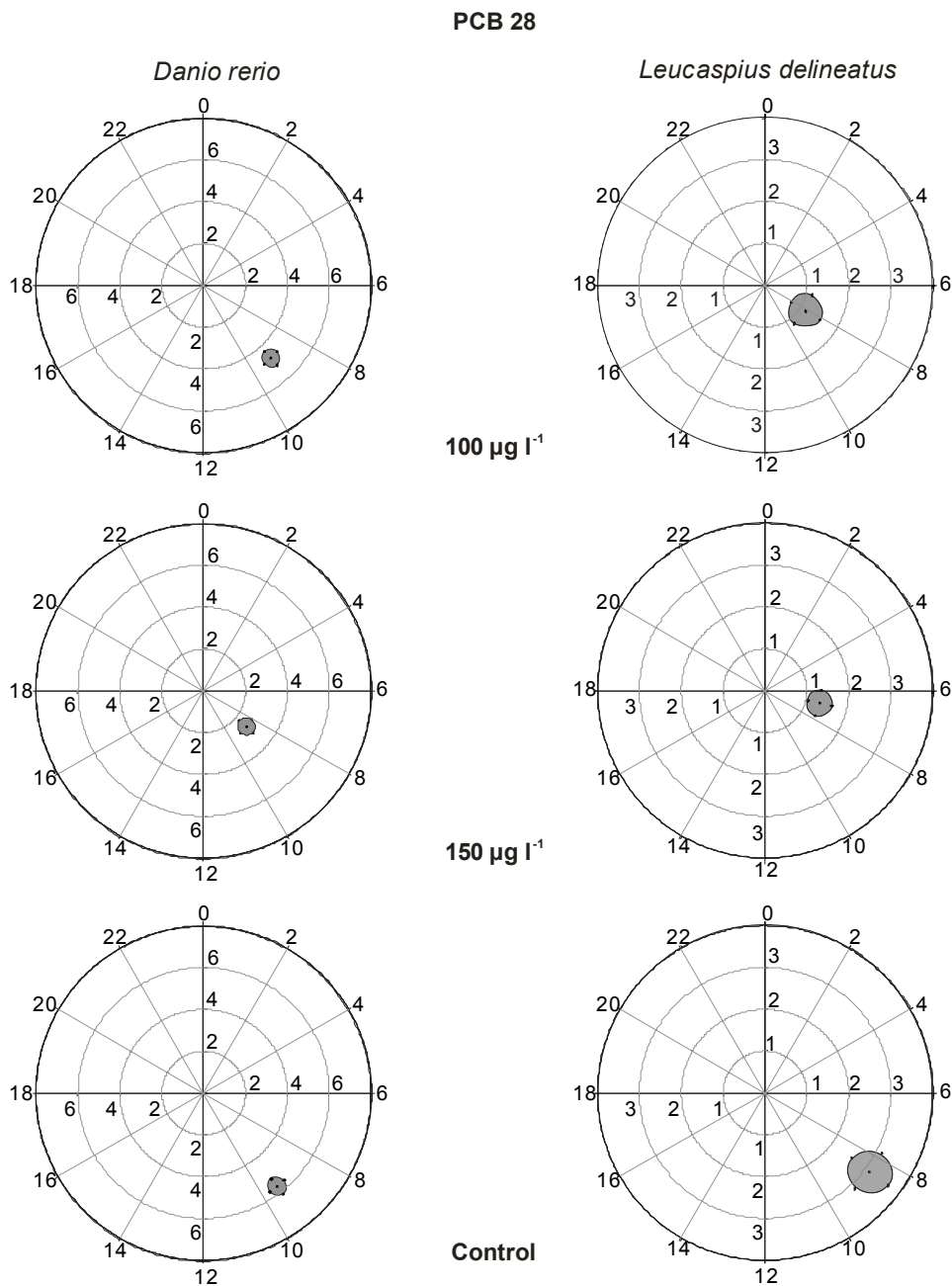


Fig. 30. Polarogram of cosinor analysis for *Danio rerio* and *Leucaspis delineatus* exposed by PCB 28. Angular axis represents the amplitude [pix s⁻¹]. Radial axis represents the time of the day [h]. Confidence ellipse covers the 95% interval.

5.2.8 Periodic frequency analysis

The activity of *Danio rerio* as well as *Leucaspis delineatus* was dominated by the 24 hours period under unexposed conditions. The 24 hours periodicity continued also under PCB 28 exposure, but the spectral power of the 24 hours component was dose related reduced compared to the control group for both fish species (Fig. 31).

For *Danio rerio* the complex of ultradian components (12, 8 and 4.8 hours period) did not change under PCB 28. In contrast, for *Leucaspis delineatus* the activity structure of ultradian components changed from formerly two significant periods of 12 and 8 hours to a single significant 12 hours period at a PCB 28 concentration of $100 \mu\text{g l}^{-1}$ and to significant 12 and 6 hours periods at a PCB 28 concentration of $150 \mu\text{g l}^{-1}$ (Fig. 31).

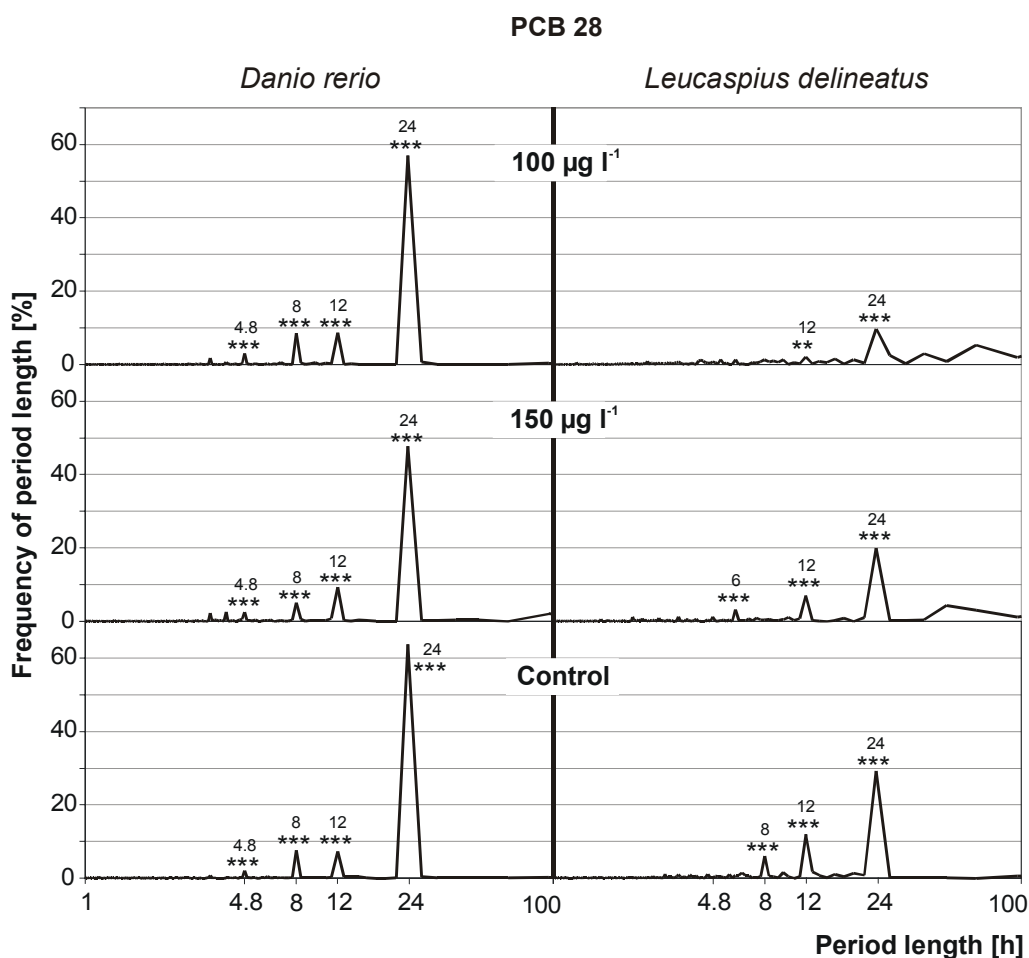


Fig. 31. Power spectrum of motility *Danio rerio* and *Leucaspis delineatus* exposed to PCB 28.

Tab. 10. Degree of Functional Coupling (DFC) and the harmonic portion (HP) of *Danio rerio* and *Leucaspilus delineatus* exposed by PCB 28.

Species	Parameter	3-PCB 28 concentration ($\mu\text{g l}^{-1}$)		
		100	150	Control
<i>Danio rerio</i>	DFC [%]	97.30	94.76	98.60
	HP [%]	80.31	69.48	83.59
<i>Leucaspilus delineatus</i>	DFC [%]	37.98	71.90	82.76
	HP [%]	11.81	30.25	48.40

For the PCB 28 experiment with *Danio rerio* the DFC was 98.6% and the harmonic portion was 83.6% for the control. The DFC values of activities and the harmonic portion decreased under exposure $100 \mu\text{g l}^{-1}$ and $150 \mu\text{g l}^{-1}$ (Tab. 10).

For *Leucaspilus delineatus* the DFC was 82.8% and the harmonic portion was 48.4% for the control. Also for this species the DFC's and the values for the harmonic portion of the exposed groups were lower compared to the control (Tab. 10).

6. Discussion

Behavioural tests addressing whole organism-level effects, may provide primary information about the hazard potential of chemicals for organisms. Focusing on the implications of a behavioural approach for ecotoxicology as branch of stress ecology, in this study the behavioural analyses were combined with chronobiological procedures such as time series analysis and power spectral analysis.

Alterations in fish behaviour including biorhythmical aspects were analysed as indicators of the sublethal toxicity of the cyanotoxin MC-LR and the xenobiotic chemical PCB 28. It was shown that dissolved MC-LR between 0.5 and 50 $\mu\text{g l}^{-1}$ and PCB 28 concentration at 100 and 150 $\mu\text{g l}^{-1}$ acted as stressors and caused significant changes in the behaviour and circadian rhythms of activity of both fish species, *Danio rerio* and *Leucaspius delineatus*.

Since sublethal effects of special stressors had to be investigated it was necessary to exclude the influence of varying environmental factors or other substances which normally occur under natural conditions. For that reason an artificial system was chosen, whereby the experimental design was characterised by a high level of invariant test conditions. Since the physico-chemical parameters of the used aquarium water were regarded to be at the normal levels of uncontaminated water for fish (see 4.2), only the added test substances (MC-LR and PCB 28) have had the potential to act as chemical stressors.

6.1 Toxicity of Microcystin-LR and Trichlorobiphenyl

6.1.1 Microcystin-LR

Several papers have shown the effects of MC-LR on fish through more or less invasive methods. In contrast to the above-mentioned effects of intraperitoneal injection and oral application (see 3.4.1.3), MC-LR in water produced no significant histopathological effects in fish (Phillips et al., 1985; Tencalla et al., 1994). Bury et al. (1995) registered an inhibited growth and disturbances of the ion balance in fish exposed to MC-LR diluted in water at concentrations of 41-57 $\mu\text{g l}^{-1}$. Rodger et al. (1994) found gill and liver damage as well as mortality for brown trout

(*Salmo trutta*) at concentrations between 16 and 19 $\mu\text{g l}^{-1}$. However in the last case the authors were not able to attribute the effects to microcystin exposure alone, as also the pH was significantly elevated and the scum of algae cells led to physical irritations of the gills.

In contrast to these results the approach of using behavioural parameters as toxicity parameters was rather sensitive. This coincides with other studies suggesting that impairment of locomotor behaviour may be a more sensitive indication for fish health than more traditional toxicity endpoints (Little and Finger, 1990; Schreck, 1990; Siegmund and Biermann, 1992; Steinberg et al., 1995; Hopkins et al., 2003). Steinberg et al. (1995) described that the lowest observed effect concentration (LOEC) of atrazine detected by behavioural analyses was more than three orders of magnitude below the acute toxicity values. A review by Little and Finger (1990) revealed that the lowest behaviourally effective toxicant concentration that induced changes in swimming behaviour of fish ranged from 0.1% to 5.0% of the LC_{50} .

The lowest observed effect concentration (LOEC) of MC-LR for behavioural and chronobiological parameters appear to be $\leq 0.5 \mu\text{g l}^{-1}$ for both tested fish species *Danio rerio* and *Leucaspis delineatus*. For MC-LR a LOEC in the same range as found for behavioural changes was determined by Oberemm et al. (1997) and Wiegand et al. (1999) applying ontogenetic and enzymatic parameters, respectively. Larvae of *Danio rerio* reared under exposure to MC-LR at a concentration of 50 $\mu\text{g l}^{-1}$ during the sensitive stages of embryonic development showed decreased survival, and larval growth and development were particularly retarded at a concentration of 0.5 $\mu\text{g l}^{-1}$ (Oberemm et al., 1997, 1999). Wiegand et al. (1999) found that the activity of biotransformation enzymes (microsomal and cytosolic glutathione-S-transferases) was elevated in zebrafish embryos, even at a toxin concentration of 0.1 $\mu\text{g l}^{-1}$.

These effects of MC-LR (including the findings of the present study) occur at environmental relevant concentrations of microcystins, associated with cyanobacterial blooms (see 3.4.1.2).

6.1.2 Trichlorobiphenyl

In the present study the non-coplanar congener PCB 28 was tested and significant behavioural and chronobiological changes were found from a concentration of

100 $\mu\text{g l}^{-1}$ upwards. In pre-tests the lower PCB 28 concentration of 50 $\mu\text{g l}^{-1}$ did not affect the behaviour of *Danio rerio* as well as *Leucaspis delineatus*.

Fingerman and Russel (1980) found effects of Aroclor 1242 on locomotor activity and on neurotransmitters (dopamine and norepinephrine) in the brain of the Gulf killifish, *Fundulus grandis*. Furthermore Aroclor 1254 led to significant changes of swimming behaviour of carp (*Cyprinus carpio*) with a LOEC of $\leq 22 \mu\text{g l}^{-1}$ (Schmidt et al., 2004). However it is not possible to judge which congeners of Aroclor are responsible for the behavioural and neurological alterations. Commercial PCB mixtures elicit a broad spectrum of toxic responses that are dependent on several factors including chlorine content, purity, dose, species and strain, age and sex of animal and route and duration of exposure (Giesy and Kannan, 2002).

Regarding behavioural parameters in fish the PCB mixture Aroclor 1254 shows a lower LOEC than the single congener PCB 28 and three causes may serve (alone or together) as an explanation: the proportion of higher chlorinated congeners, the proportion of coplanar (dioxin-like) congeners and the potential interactions between the single components of the mixture. The toxicity of a specific PCB congener is dependent upon both its degree of chlorination and the position of its chlorine atoms (Safe, 1984). Less chlorinated PCBs are lesser bioconcentrated and more readily metabolised and excreted (Giesy and Kannan, 2002). For PCB 28 no accumulation in the brain of fish was found (Qi et al., 1997).

Toxic effects due to coplanar PCBs occur at relatively smaller concentrations than those due to non-coplanar PCBs (Giesy and Kannan, 2002). For example in rainbow trout (*Oncorhynchus mykiss*), coplanar congeners affected early life stage mortality (Walker et al., 1996), in contrast to non-coplanar PCBs that did not induce early life stage mortality (Walker and Peterson, 1991; Hornung et al., 1996). However the main portion (81%) of Aroclor 1254 consists of non-coplanar PCBs (Frame et al., 1996), thus there is only a modest amount of coplanar PCBs in Aroclor 1254.

Interactions among individual PCB congeners were found by Seegal et al. (1990), whereby a mixture of three non-coplanar PCBs (one of them was PCB 28) was more potent in reducing brain dopamine content than the equal amounts of each congener in tissue cultures of nonhuman primate brain. A further study found that three single non-coplanar congeners did not cause any toxic effects on embryos and newly hatched larvae of the Japanese medaka (*Oryzias latipes*), but all three

together caused an inhibition of swim bladder inflation in the newly hatched larvae (Kim and Cooper, 1999).

Another uncertainty associated with the assessment of toxicity based on exposure to commercial PCB mixtures is related to the relative amounts of polychlorinated dibenzofurans (PCDFs) and polychlorinated naphthalenes (PCNs) identified as contaminants in technical PCB preparations (Giesy and Kannan, 2002), whereby dioxin-like potencies of polychlorinated naphthalenes were found by Blankenship et al. (2000).

Therefore, for analysing relations between structure and toxic effects of PCBs it is useful to separately determine the toxicity of single congeners. This is moreover important, because of the qualitative differences between technical mixtures of PCBs and the mixtures occurring in the environment (see 3.4.2.2). Since the susceptibility of fish to Aroclor 1254 was clearly higher compared with effects of the single congener PCB 28 tested in the present study, PCB 28 cannot be the congener which led to the major toxic effects of PCBs. However, combined effects of this single congener with other PCB congeners can not be excluded.

6.2 Fish-species specific differences to chemical stressors

Inherent sensitivity varies among species and chemicals because of the differences in the absorption (the amount of chemical entering the organism), translocation (movement of the chemical within the body), and biotransformation (metabolic activation, detoxification and excretion) of chemicals in relation to the presence of target and non-target receptors (Grue et al., 2002). However publications in which the susceptibilities of different fish species to water contaminants are compared are scarce. Physiological and behavioural responses to the same chemicals may differ markedly between species, for example the level of locomotor activity of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) differed by one order of magnitude under the influence of zinc (Sprague, 1964; Sprague, 1968). If individuals or species differ in their responses to a particular gradient of an abiotic factor, changes in intra- or interspecific interactions can be expected. For example, the greater sensitivity in the avoidance response of rainbow trout (*Oncorhynchus mykiss*) to metal contamination compared with brown trout (*Salmo trutta*) may

explain observed differences in the distributions of the two species in the Clark Fork River in Montana (Hansen et al., 1999).

Some publications are concerned with fish species specific differences of susceptibilities to cyanotoxins. In an acute toxicological study Bury et al. (1997) observed that brown trout (*Salmo trutta*) was less tolerant to MC-LR than rainbow trout (*Oncorhynchus mykiss*). From recent studies, it is known that some species from temperate lakes are highly sensitive to cyanotoxins e.g., whitefish (*Coregonus lavaretus*) (Ernst, 2001), whereas species from subtropical lakes are able to use it as food e.g., silver carp (*Hypophthalmichthys molitrix*), (Xie et al., 2004). Xie et al. (2004) found no detectable microcystin in the muscle and blood of silver carp (*Hypophthalmichthys molitrix*) in contrast to previous experimental results on rainbow trout (*Oncorhynchus mykiss*) and argue that silver carps have a mechanism to degrade or eliminate MC-LR actively and to inhibit its transportation across the intestines. Since both *Danio rerio* and *Leucaspius delineatus* showed highly sensitive reactions to MC-LR in the present study there is no hint for such an elimination mechanism.

For PCBs species specific differences were found in the extent of bioaccumulation for three estuarine fish species (red mullet *Mullus barbatus*, sea mullet *Mugil cephalus* and sea bass *Dicentrarchus labrax*) from the same area, whereby the extent of bioaccumulation was dependent on lipid content, habitat, dietary intake, growth rate and the metabolism rate of each species (Pastor et al., 1996). Significantly higher levels of PCBs were shown for slow growing Arctic char (*Salvelinus alpinus*) than for fast growing char (Hammar et al., 1993). Furthermore significantly higher mean tissue concentrations of Aroclor 1260 were found in predator species (0.23 ± 0.38 ppm) compared to bottom feeders (0.14 ± 0.24 ppm), whereas lower chlorinated Aroclors (1248 and 1254) did not lead to significant differences in residues in bottom feeding and predatory fish (EPA, 1999). Applying an individual-based model (IBM), the lower observed PCB concentrations in rainbow trout (*Oncorhynchus mykiss*) compared with lake trout (*Salvelinus namaycush*) from one lake were explained by the greater longevity in lake trout and the observed variation in PCB accumulation rate within the population of rainbow trout was explained by variation in prey PCB concentrations (Madenjian et al., 1994).

In the present study the two fish species *Danio rerio* and *Leucaspius delineatus* were selected in order to analyse potential specific differences in effects from sub-

lethal exposure to MC-LR and PCB 28. Because of its uncomplicated rearing and reproduction the species *Danio rerio* is often used in toxicological research. To compare the effects of *Danio rerio* with an endemic species the sensitive and partially endangered species *Leucaspius delineatus* was investigated. Since the observed differences in the reactions of both species to MC-LR and PCB 28 were rather small, the results of the species *Danio rerio* are comparable to those of the native European species *Leucaspius delineatus*. Some possible reasons could be the similar systematic origin from the family Cyprinidae, comparable size and weight and age (see 4.1).

6.2.1 Effects on locomotor activity of *Danio rerio* and *Leucaspius delineatus*

Within the context of different behavioural responses, locomotion plays an important role because it is an integrative indicator of the internal status of the animals. The usefulness of locomotor responses in behavioural ecotoxicology is based on the fact that they are objective and automatically quantifiable and can be measured effectively in a variety of fish species to characterise the consequences of sublethal exposures (Little et al., 1993).

The experimental system, that was used to observe and report the fish behaviour, was an artificial one. Beside its above-mentioned advantages there are restrictions, most obvious, the limited volume of the aquaria.

Interestingly despite this relatively small volume the method allowed not only the description of the swimming velocity but also of the swimming mode of fishes. So the relationship between number of turns and motility clearly indicated species specific differences (see 5.1.5 and 5.2.5). This implied that the pattern of swimming activity were not generally determined by the limited volume of the aquaria.

6.2.1.1 Microcystin-LR induced behavioural effects

Some changes in the spontaneous locomotor behaviour of both fish (*Danio rerio* and *Leucaspius delineatus*) suggest that both species have a comparable susceptibility to MC-LR in this study. Whereas lower concentrations increase the motility, the highest exposure concentration, 50 µg l⁻¹, caused a significant decrease in activity of both *Danio rerio* and *Leucaspius delineatus*. That corresponds well to the hormesis theory (see 6.3.1).

However, some differences between the two species were found in their reaction to MC-LR. So analysis of mean motility including exposure-time dependent aspects showed that *Leucaspilus delineatus* reacted earlier (at MC-LR concentrations of 0.5, 5, and 15 $\mu\text{g l}^{-1}$) and for a longer time (at 50 $\mu\text{g l}^{-1}$) than did *Danio rerio*. Along with motility, turns characterise the swimming activity changes. The numbers of turns were significantly increased during the night phase at all MC-LR concentrations for *Leucaspilus delineatus* and at elevated MC-LR concentrations for *Danio rerio*. Furthermore the regression analysis between motilities and turns was indicative for MC-LR induced effects on swimming performance of fish. The statistic relation (r^2) between the increase in number of turns and increasing motility was weaker at elevated concentrations of MC-LR compared to the control for *Danio rerio* and, however, was stronger compared to the control for *Leucaspilus delineatus*. Additionally for *Danio rerio* the significantly decreased slope of the regression at the highest MC-LR concentration of 50 $\mu\text{g l}^{-1}$ indicated that the fish decreased the number of turns at a given motility and swam more smoothly. In contrast, for *Leucaspilus delineatus* the slope of regression increased under the influence of MC-LR indicating the fish swam more jerkily under exposure.

6.2.1.2 Trichlorobiphenyl induced behavioural effects

Some reactions of *Danio rerio* as well as *Leucaspilus delineatus* exposed to PCB 28 are very similar, as it was shown for the MC-LR induced reactions. Elevated exposure concentrations of PCB 28 (150 $\mu\text{g l}^{-1}$) caused a significant decrease of activity in *Danio rerio* as well as in *Leucaspilus delineatus*. Analysis of mean motility including exposure-time dependent aspects showed that both *Danio rerio* and *Leucaspilus delineatus* reacted to the highest PCB 28 concentration (150 $\mu\text{g l}^{-1}$) from the first to the last exposure interval. Beside those similarities, PCB 28 led to some changes of spontaneous locomotor behaviour of both fish species which suggest some differences between *Danio rerio* and *Leucaspilus delineatus*. At the lower PCB concentration of 100 $\mu\text{g l}^{-1}$ the mean motility over the whole measuring time per day did not change significantly for *Leucaspilus delineatus*, whereas the activity level of *Danio rerio* was significantly reduced. However differentiating the activity rhythms of *Leucaspilus delineatus* according to daylight and nighttime activity significant effects of PCB 28 at the lower PCB concentration (100 $\mu\text{g l}^{-1}$) were found, too.

Indicated by the analysis of exposure-time dependent aspects *Danio rerio* reacted more rapidly and over a longer time period than did *Leucaspilus delineatus* at PCB concentrations of $100 \mu\text{g l}^{-1}$. Significant changes of motility of *Leucaspilus delineatus* were only recorded in the second exposure interval and afterwards disappeared from the third exposure interval up. The numbers of turns were significantly decreased during the daylight phase at PCB 28 concentrations of 100 and $150 \mu\text{g l}^{-1}$ for *Danio rerio*, and only at elevated concentrations ($150 \mu\text{g l}^{-1}$) for *Leucaspilus delineatus*.

The regression analysis between number of turns and motility indicated similar statistic relations under exposure of PCB 28 or control conditions for *Danio rerio*. In contrast, for *Leucaspilus delineatus* the statistic relation between the increase of number of turns and increasing motility was stronger and the slopes of the regressions were significantly increased under PCB 28 exposure. Therefore, only for *Leucaspilus delineatus* the variability of the pattern of swimming performance was decreased and the kind of swimming performance was changed in that kind that the fish swam more jerkily under the influence of PCB 28.

6.2.2 Effects on chronobiology of *Danio rerio* and *Leucaspilus delineatus*

Analysis of temporal patterns in biological data gives insight into acclimation processes. If the fish are well acclimated to the experimental conditions they will obtain stable rhythms of locomotor activity synchronised by external rhythms, e.g., by temporal variations of light and food (e.g., Schwassmann, 1980; Boujard and Leatherland, 1992; Siegmund and Biermann, 1992). In contrast, disturbance may lead to a desynchronisation between biological rhythms and their zeitgeber, whereby if the peaks of activity are occurring later (delay) or earlier (advance) in comparison to unexposed conditions the rhythm is phase shifted.

6.2.2.1 Cyclic aspects of behaviour under unexposed conditions

Since the swimming activity of fish may show distinct day/night differences (e.g., Plaut, 2000; Campbell et al., 2002) biorhythmic aspects have to be necessarily considered in behavioural studies. Under unexposed conditions both fish species of this study *Danio rerio* and *Leucaspilus delineatus* exhibited a significantly diurnal activity (indicated by the effects of zeitgeber) which coincides with other stud-

ies dealing with the circadian periodicity of *Danio rerio* (Baganz et al., 1998; Hurd et al., 1998; Plaut, 2000) and *Leucaspius delineatus* (Siegmond & Wolff, 1973). The daily activity of both fish species was synchronised with the artificial alternation of light and dark and the feeding times within the 24 hours period. The maximum of daily activity of *Danio rerio* as well as *Leucaspius delineatus* was registered shortly after the onset of light. This was the time period of the daily mating and spawning behaviour of *Danio rerio*.

Spectral analysis of activity in both fish species showed a time pattern which was characterised by a dominant 24 hours rhythmicity, but also by ultradian components with period length between 4.8 and 12 hours. Single cosinor analysis revealed significant circadian rhythms with equal periods of $24 \text{ h} \pm 1.14 \text{ h}$ for both fish species, whereas the values of amplitude and MESOR were higher for *Danio rerio* than for *Leucaspius delineatus*.

6.2.2.2 Microcystin-LR induced chronobiological changes

There were some similar reactions of *Danio rerio* and *Leucaspius delineatus* in response to MC-LR exposure. In both fish species a degree of desynchronisation to the light/dark change was found which led to a phase shift in *Danio rerio* as well as in *Leucaspius delineatus* indicating that the influence of the zeitgeber light decreased under exposure to the toxin. This was expressed by the reduced value of the Degree of Functional Coupling (DFC) which is an objective parameter of the coordination of different organismal functions both with each other and with the external circadian zeitgeber (Scheibe et al., 1999). A reduced value of DFC for activity of alpacas (*Lama guanicoe f. pacos*) was for instance found for an accidentally hurt animal in comparison with healthy animals by Scheibe et al. (1999).

Further stress symptoms caused by MC-LR (at a concentration of $50 \mu\text{g l}^{-1}$) were the reduced amplitude which was significant only for *Danio rerio* and the significantly decreased MESOR for *Danio rerio* as well as *Leucaspius delineatus*. The latter one shows the drastically reduced activity level in both fish species. The significantly increased MESOR or amplitude at lower exposures reflect a significant increase in daytime motility.

However there were some differences between the two test species concerning the observed phase shift which is graphically represented in the polar plots. For *Danio rerio* a phase delay occurred, whereby at all concentrations the changes could

only be registered during the light phase. Therefore, *Danio rerio* remained still significantly diurnally active (indicated by the effects of zeitgeber). In previous studies of the author it was shown that the phase delay was associated with circadian variations during the daily spawning time and an evidently reduced reproduction success at an MC-LR concentration of $50 \mu\text{g l}^{-1}$ (Baganz et al., 1998). In contrast, the phase of *Leucaspilus delineatus* advanced, whereby this shift was so drastically that a phase reverse occurred and this species became significantly nocturnal as indicated by the effects of zeitgeber (for considerations of ecological effects see 6.3.3). Comparable changes in the circadian periodicity of fish activity (phase shift of activity, degree of desynchronisation to the light/dark change) induced by nitrogen compounds were found e.g., by Biermann (1992).

Performing cosinor analysis on motility of *Danio rerio* resulted in distinctive single peaks and a stable period length of circadian rhythms for all tested MC-LR concentration as well as the control. However, for *Leucaspilus delineatus* these single peaks of circadian motility rhythms were found only at the control and the lowest applied MC-LR concentration. Interestingly, at elevated MC-LR concentrations clear double peaks of circadian motility rhythms of *Leucaspilus delineatus* occurred, indicating two oscillations of shorter periods. These two main oscillations were found by the power spectrum analysis of *Leucaspilus delineatus*, too, whereby the dominance of the circadian rhythmic peak was clearly reduced, and simultaneously, the proportion of a harmonic oscillation with a 12 h rhythm increased under the influence of higher concentrations of MC-LR. Because of this increased amount of ultradian rhythms, the period length calculated by cosinor analysis as the mean value of all frequency parts significantly decreased for *Leucaspilus delineatus*. In contrast to *Leucaspilus delineatus*, for *Danio rerio* the ultradian rhythms were more affected than the circadian component by MC-LR exposure. The same effect was found by Scheibe et al. (1999) for alpacas (*Lama guanicoe f. pacos*) under stress conditions. These findings emphasize the importance of the circadian rhythmic component.

6.2.2.3 Trichlorobiphenyl induced chronobiological changes

Similar reactions of *Danio rerio* and *Leucaspilus delineatus* in response to PCB 28 (at a concentration of $150 \mu\text{g l}^{-1}$) were reflected by a significantly reduced amplitude as well as MESOR. The reduced MESOR indicates the reduced activity of

both species (influenced by PCB 28) over the whole measuring time per day. The values of the effects of zeitgeber were significantly reduced for *Danio rerio* as well as *Leucaspilus delineatus*, whereby both species remained diurnally active with a stable period length under exposure. In contrast, some effects on cyclic aspects of behaviour clearly indicate that *Leucaspilus delineatus* reacted more sensitively to PCB 28 than *Danio rerio* did. A degree of desynchronisation to the light/dark change (indicated by the reduced values of DFC) which led to a phase advance, was only found for *Leucaspilus delineatus* under exposure of PCB 28. Furthermore only for *Leucaspilus delineatus* the dominance of the circadian rhythmic peak was reduced whereas for *Danio rerio* the complex of ultradian components did not change under PCB 28 exposure.

6.2.3 Stress potential of Microcystin-LR and Trichlorobiphenyl

Taken together, the analysed parameters gave clear evidence that both the cyanobacterial toxin MC-LR as well the xenobiotic chemical PCB 28 revealed stress symptoms on the behavioural and chronobiological level in fish occurring under sublethal conditions.

Most of the changes in level and rhythms of swimming activity of both fish *Danio rerio* and *Leucaspilus delineatus* suggest a comparable susceptibility to MC-LR as well as PCB 28. For MC-LR it could be shown that the reaction of *Leucaspilus delineatus* was a bit more sensitive than of *Danio rerio*. For PCB 28 the analysis of the activity level indicated that *Danio rerio* tended to be more sensitive than *Leucaspilus delineatus*, whereas the registered rhythmical parameters indicate that *Leucaspilus delineatus* reacted in this respect more sensitively to PCB 28 than *Danio rerio* did.

Some reactions of the *Danio rerio* as well as the *Leucaspilus delineatus* were independent from the kind of chemical stressors (MC-LR and PCB 28), despite of their different chemical and physical properties. That means that the behavioural parameter responds non-specifically (but very sensitive) to any toxic chemicals. So, for both species elevated concentrations of the stressors led to a reduction of the activity.

Furthermore the rhythmical parameters (MESOR, amplitude) changed significantly under exposure of both substances (MC-LR or PCB 28), as indicated by the cosinor analysis. Analysis of the degree of synchronisation between activity

rhythms and their zeitgeber (by power spectral analysis, DFC values and effects of zeitgeber) as well as the quantification of the harmonic frequency structure of activity rhythms (by power spectral analysis) proved to be good indicators for environmental changes, corresponding to findings of Siegmund and Biermann (1989, 1990) and Scheibe et al. (1999) (see 6.2.2).

For *Leucaspius delineatus* both MC-LR as well as PCB 28 led to an increase of the slope of regression between number of turns and motility indicating that the swimming patterns were influenced in the same direction independently from the kind of stressor. However this could not be verified for *Danio rerio*, since PCB 28 did not affect the swimming patterns in contrast to MC-LR. The phenomenon of stressor-induced change of swimming mode has to be examined for its relevance in further studies.

6.3 Applications of behavioural approaches in ecotoxicology

This thesis is engaged in the recently still emerging field of behavioural ecotoxicology which integrates the three different disciplines: ethology, toxicology and ecology (Dell'Omo, 2002). Because the behaviour is the cumulative manifestation of genetic, biochemical, physiological and environmental cues, behavioural data may provide a link between individual response and population change, especially for those behaviours that manifest ecologically as changes in structure and function of the community (Little et al., 1985).

Toxicant-induced changes in behaviour may indicate toxicity (the failure of adaptive mechanisms) or conversely may be the adaptive response of an animal to mitigate or obviate the potential effects (Dell'Omo, 2002). Since fish have coevolved with cyanotoxins over phylogenetical long periods it could be hypothesized that protective adaptive mechanisms have developed. The fast and sensitive behavioural reactions found in this study support this theory. On the other hand it is rather unlikely that appropriate adaptive responses on the behavioural level to anthropogenic contaminants that are recent additions to the environment, have developed.

Basic knowledge of exposure related behavioural alterations relevant for ecotoxicological assays remain scarce, and systems that have the ability to link toxicology data with swimming behaviours are still needed (Vogl et al., 1999).

The research on fish behaviour as an indicator of toxic effects is currently getting more and more attention (e.g., Chon 2002) and the findings in this study indicate that the non-invasive automatic registration of activity data is a suitable approach on the way to more sensitive ecotoxicological research methods and practicable for a range of applications.

Locomotor activity as a main component of behaviour is generated and controlled by different physiological processes and motivational states of an organism. Since it is important for such activities as feeding, predator avoidance, competitive interactions, migration behaviour (Reidy et al., 2000) it is also relevant in the ecological context. However one should be careful not to overinterpret effects found in the laboratory (Zala and Penn, 2004) since in natural ecological setting, individuals might be able to avoid the source of exposure or develop tolerance to pollutants (Barron, 2002).

6.3.1 Hormesis theory

It has long been assumed that chemicals have a threshold level of safe exposure, and that dosage effects are linear; however, these assumptions have turned out to be obviously incorrect. Among others, Calabrese and Baldwin (2003) established the hormesis theory as a fundamental new concept in toxicology used to determine risks and risk regulations. According to these authors hormesis is a dose-response relationship phenomenon characterized by a stimulation of response at low doses and an inhibition of response at high doses.

Chemical stressors that elicit hormesis in organisms may induce therefore more than one mode of action, resulting in either a J-shaped or an inverted U-shaped response. Hormetic responses have been reported in hundreds of studies for a broad range of species (protozoa, bacteria, fungi, plants, invertebrates and vertebrates including humans), biological endpoints (e.g., survival, growth, reproduction), and both inorganic and organic chemicals (Calabrese and Baldwin, 1997). Therefore, such non-monotonic effects (or hormesis) are the rule rather than the exception in toxicology studies. Hormesis was also found on the behavioural level, e.g., male scent-marking behaviour increased when mice during foetal life were exposed to low doses of estrogenic pesticides (methoxychlor, DDT and a synthetic oestrogen (DES)), but marking behaviour declined again at the highest dose of DES (vom Saal et al., 1995). A stimulatory effect of a sublethal, light anaesthetic

dose of tricane methane sulfonate (MS222) on velocity of mummichog (*Fundulus heteroclitus*) was observed by Kane et al. (2004).

Some of the dose-responses registered in the present study correspond to the hormesis theory: there was an increase of daytime activity level at lower MC-LR concentrations and a decrease of these effects at elevated concentrations of MC-LR for both *Danio rerio* and *Leucaspius delineatus*. Another hint of hormesis in the present study was the increase of the proportion of the 24 hours periodicity related to the whole harmonic frequency structure at lower MC-LR concentrations whereas this proportion decreased at elevated concentrations for *Danio rerio*. That means the synchronisation between activity rhythms and their zeitgeber was stronger at lower MC-LR concentrations and weaker at higher MC-LR concentrations compared to the control.

However, at moderate concentrations no significant effects of MC-LR on the daytime motility were observed for both fish species. Furthermore the power spectrum of MC-LR concentrations of $15 \mu\text{g l}^{-1}$ is equal to that of the control for *Danio rerio*. Such an absence of a biological effect at moderate doses of a chemical (imazalil sulphate) on growing populations of the green algae *Scenedesmus quadricauda* was as well observed by Prokhotskaya et al. (2000), whereas lower concentration resulted in an increase of cell number and higher concentrations resulted in cell death.

Alternatively, the fact that PCB 28 did not elicit hormetic responses may be indicative for a single mode of action caused by higher levels of stressor exposure. According to the hormesis theory the inhibition follows often an initial stimulatory response, appearing to represent a modest overcompensation of a disruption in homeostasis. However, higher exposures may exceed the capacity to compensate and lead to an adverse response compared with the controls.

The following explanations for the phenomenon of hormesis were summarized by Prokhotskaya et al. (2000): Weak treatments impair the regulatory mechanisms, what leads to an activation of regulated processes. The further increase in the factor strength disturbs the functioning of regulated systems and suppresses metabolic reactions, which can result in cell death. Another explanation is based on the assumption that weak treatments induce detrimental changes in the cell qualitatively similar to those induced by stronger treatments, although to a lesser extent. Cell response involves protective mechanisms which, in addition to compensating

for impairments, can result in hypercompensation. Stimulation of membrane receptors is believed to be a cause for the activation of cell functions. Under stronger actions, the activity of membrane receptors is suppressed.

From the ecotoxicological point of view it is important to determine whether hormesis has a positive, neutral or adverse effect on the overall health of organisms. Positive effects of hormesis e.g., on the longevity are demonstrated by some authors (Neafsey, 1990). So hormesis is an active survival strategy of organisms under altered conditions (Prokhotskaya et al., 2000).

6.3.2 Behavioural strategies

The strategies used by organisms to resist chemical exposure can be classified into several general categories: avoidance or escape reactions, exclusion (for example, many aquatic animals exposed to toxic chemical secrete mucus onto exposed surfaces), removal (in-coming toxicants might be actively pumped out), detoxification, possibly followed by excretion (e.g., by sequestration in granules or via metabolic transformation), and repair of damage caused by toxicants (Forbes and Calow, 1996). Therefore, the increase in daytime motility of *Danio rerio* and *Leucaspis delineatus* at lower MC-LR exposures in the present study can be interpreted as an escape reaction and/or as an increased spatial orientation behaviour. Tembrock (1984) described spatial orientation as an active behavioural adaptation to cope with changing ecological conditions. However increases of activity are costly for the organisms in terms of metabolic resources and especially energy. This energy needed for faster swimming is not available for other living processes, e.g., growth or reproduction. Thus, the determination of whether the low dose stimulation (according to the hormesis theory) is beneficial or harmful is not always obvious and must be judged in every single case.

Increased MC-LR concentration caused significant decreases in motility of both species over the whole measured time per day. Hence an escape and/or orientation behaviour was not any more the dominant strategy under increased exposure. It is hypothesized that physiological disruptions (connected with an increased energy demand) are responsible for the observed effects, whereby at the point of maximum achievable metabolic scope under these conditions the amount of surplus energy available for swimming was supposed to be significantly decreased. This assumption was supported by the findings of some studies which found signifi-

cantly lowered swimming speeds in fish exposed to sublethal concentrations of toxicants (McGeer et al., 2000; Campbell et al., 2002; Hopkins et al., 2003). Certainly at least a part of the saved energy is needed for the biotransformation process of the toxins.

Organisms are able to metabolise toxins by oxidation, reduction and hydrolysis (phase 1; catalysed e.g., by the cytochrome enzyme P450 monooxygenase (CYP 1A)), and by conjugation (phase 2, e.g., via glutathione-S-transferase).

The biotransformation process of MC-LR by elevation of microsomal and cytosolic glutathione-S-transferase activity, was described by Pflugmacher et al. (1998), who identified in various aquatic organisms including fish an enzymatically formed glutathione conjugate of MC-LR as the first step of biotransformation and its degradation to a cysteine conjugate. An elevated activity of biotransformation enzymes: microsomal and cytosolic glutathione-S-transferase; glutathioneperoxidase in zebrafish embryos was found by Wiegand et al. (1999).

Exposure to PCBs led to a significant elevation of the cytochrome enzyme P450 monooxygenase (see chapter 3.4.2.4) as well as of the conjugation enzyme system (cytosolic glutathione-S-transferase) in fish (Koponen et al., 2000; Schmidt 2004). PCB biotransformation has been shown to lead to two classes of PCB metabolites that are present as contaminant residues in the tissues of selected biota: hydroxylated (HO) and methylsulfone (MeSO₂) PCBs (Letcher et al., 2000). Hydroxylated PCBs in lake trout (*Salvelinus namaycush*) blood plasma were found by Campbell et al. (2003). Both hydroxylated and methylsulphonyl metabolites of PCBs are reported to be toxic (Brouwer et al., 1997).

The significance of energy saving behaviour under exposure is also expressed by compensation processes between daylight and night activity. So it was shown that for *Danio rerio* increases in daylight activity from MC-LR exposure (at 0.5 µg l⁻¹) were compensated for, at least partly, by decreases in activity at night. The same applied to *Leucaspius delineatus*, whose decrease in daylight motility (at MC-LR concentrations of 15 µg l⁻¹ and 50 µg l⁻¹; at PCB 28 concentration of 100 µg l⁻¹) corresponded with a motility increase in the dark. Obviously, this activity shift compensates the elevated energy expenditure during the metabolic more active phase. Therefore, the data show the importance of evaluating activity rhythms differentiated according to daylight and nighttime activity, while analysing only the

average activity over the days span would minimize the effects of the stressor by levelling out the phase related effects.

Reducing the activity (and the costs that go along with it) can be an effective strategy if exposure is of temporary nature and the energy made available for stress decreasing processes (e.g., enzyme production) is sufficient to overcome this period. However a lowered activity over longer-term exposures is likely to indicate an impaired performance e.g., in the form of reduced feeding and mating activity (Forbes and Calow, 1996) and may lead to a metabolic collapse in the organism. In previous studies the reduced activity level and the phase shift of activity of *Danio rerio* at the MC-LR concentration of $50 \mu\text{g l}^{-1}$ coincided with a reduced spawning activity and success (Baganz et al., 1998). The species *Leucaspis delineatus* reproduces in April-May near shorelines and among vegetation where in meso- to eutrophic lake blooms of cyanobacteria may occur because of, for example, wind and current, and therefore an impact on its spawning behaviour is possible. However it is difficult to synchronise the reproduction of *Leucaspis delineatus* under laboratory conditions since the reproduction of this species is limited to a special period of the year.

Because swimming performance is of central importance for many aspects of fish biology, their reduction following exposure to contaminants could ultimately diminish the fitness in affected individuals and have consequential implications for inter- and intraspecific interactions (Hopkins et al., 2003). Plaut (2000) pointed out that a reduction in swimming capability, resulting in a reduction in the rate of activity, may decrease the ability to gather food, making the fish vulnerable to predation. Impairments in foraging behaviour of mummichogs (*Fundulus heteroclitus*) from contaminated sites appeared to have accounted for their reduced growth and longevity in comparison to those from uncontaminated sites, whereby grass shrimps, which are an important prey species of the mummichogs, had a greater population density and a larger size-frequency distribution at the polluted site, apparently because of reduced predation pressure (Weiss et al., 1999).

6.3.3 Chronobiological analysis

Quantifying stress induced behavioural changes of continuous recordings facilitated the registration of rhythmical changes and provided therefore novel information on the stress potential of the investigated contaminants. Time patterns in

swimming activity showed an at least as sensitive response to the chemical stressors, as it was shown for the basic behavioural parameters. So the LOEC-value for rhythmical parameter was in the same range as for basic behavioural parameter for both MC-LR as PCB 28.

With finding a mathematical equation for modelling the circadian activity rhythms of fish over a given time period by using the cosinor analysis it was possible to explain the substantial proportion of data which inherited a 24 hours periodicity.

The power spectral analysis using the program "Zeit" (Scheibe et al., 1999, 2002) with their amplitude coefficients gives a measure of how well the activity rhythms of fish fit infradian, circadian and ultradian sinusoidal frequencies. The remaining proportion of data was only the white noise, that means the part of the raw data which inherited no more significant rhythmic components (StatSoft, Inc., 2004).

Deferment of phase relations, changes in frequency structure, loss of rhythmicity or reduction of amplitude are regarded as signs of adaptation, disease or pre-mortal state (Scheibe et al., 1999). For analysing changes of circadian rhythms of humans caused by diseases (e.g., Alzheimer's Disease) some studies deal with an evaluation of various rhythmic parameters like MESOR, amplitude, acrophase and period (e.g. Volicer et al., 2001). For some species of hoofed animals the ultradian rhythms of activity were more affected than the circadian component by external disturbances using the power spectral analysis (Berger et al., 2002, 2003). However these utilised analytical methods of biorhythmic research have rarely been applied for estimating the risks of aquatic contaminants, and it would be useful to bring them more into focus of ecotoxicology. Methods of biorhythm research require continuous activity records over extended periods of time.

Since the response of rhythmic changes seems to be a characteristic reaction of fish exposed to waterborne toxicants, it is a good indicator of sublethal stress (Siegmund & Biermann, 1990). This has been verified by investigations on fish in the present study, whereby the results proved that the extended methods of time series investigation like cosinor analysis and power spectral analysis can be valuable tools for the study of harmful environmental factors. In the present study the amount of ultradian rhythms of *Leucaspius delineatus* were clearly increased under MC-LR exposure.

The DFC which is an objective parameter of coordination of different organismal functions both with each other and with the external circadian zeitgeber, enables

an assessment of the organismal state (Scheibe et al., 1999). Analysis of DFC in the present study indicates that the synchronisation between circadian rhythms of activity and their external zeitgeber was weaker under the influence of the chemical stressor (see 5.1.8 and 5.2.8). The reduction of the synchronising effects of light on the locomotor activity rhythm in both *Danio rerio* and *Leucaspilus delineatus* was partly accompanied by a shift of the most active period of the day to another time period of the day.

Disturbances of that equilibrium (the homeostasis) can be answered by a variety of integrative reactions (biochemical, physiological, behavioural) with different priorities. The high flexibility of biological rhythms (Aschoff 1984) results in a rather early alteration of circadian periodicity to deal with stress conditions.

According to the above mentioned enhanced spatial orientation behaviour under exposure, the observed phase shift of behavioural rhythms in both *Danio rerio* and *Leucaspilus delineatus* species could be interpreted in a similar way as an enhanced or new temporal orientation. Rhythmicity has a great adaptive value for the precise temporal fit of the organism into the ecosystem (Tembrock, 1992), the ecological niche has therefore not only a spatial but also a temporal component.

Because various environmental conditions oscillate cyclically, the ability to anticipate temporal changes in the environment would enable an organism to be prepared, both physiologically and behaviourally, to perform specific activities when the environmental conditions are most favourable to the species (Hoenen & Gnaspini, 1999). Therefore, the phase shift of behavioural rhythms under exposure may lead on the one hand to physiological disturbances of the temporal coordination of internal processes (internal desynchronisation; Aschoff, 1969). On the other hand it may lead to interspecies disadvantages under natural conditions, e.g., in the context of efficiently searching for prey and avoiding predators.

As mentioned above biorhythmic changes can also come along with a reduced reproduction success. In fishes it was demonstrated that feeding time affects growth performance (Boujard and Leatherland, 1992; Bolliet et al., 2001) which indicated the importance of synchronisation between external rhythmical events and internal processes. It can be assumed that a variety of behavioural and physiological rhythms which are usually coupled become dissociated under stress conditions.

Such a dissociation between circadian rhythms of swimming activity and heart rate of fish indicated harmful effects on fish caused by temperature stress (Sieg-

mund, 1981). Disturbances of coordination between respiratory-cardiovascular processes are used for identifying acute toxicity syndromes of rainbow trout (*Oncorhynchus mykiss*) (McKim et al., 1987). Bolliet et al. (2004) suggests that physiological rhythms involved in nutrient utilisation might not be as flexible as feeding rhythm thereby leading to a desynchronisation between rhythms and possibly a decrease in growth and feeding efficiency.

The ability to predict a regularly occurring environmental change accurately and consistently and anticipate the necessary behavioural adjustments is critical to the survival of a population (Adgins-Regan and Weber, 2002). Johnson et al. (2003) addressed the adaptive significance of circadian rhythmicity by testing the relative fitness under competition between various strains of cyanobacteria expressing different circadian periods, whereby strains that had a circadian period similar to that of the light/dark cycle were favoured under competition.

The effects of MC-LR and PCB 28 were more drastically at the time of switching the light on or off as it was shown by the smoothed curve of average motility. Daily variations in sensitivity of fishes to harmful stimuli were also shown by Spieler et al. (1977), whereby fathead minnows (*Pimephales promelas*) and golden shiners (*Notemigonus crysoleucas*) exhibited differences in sensitivity to potentially lethal levels of chlorine, formalin, or heat, depending on the time of day. Therefore, the meaning sense and the reliability of behaviour measurements to recognise stressful impacts can be enhanced by a chronobiological analysis of data, as confirmed by several authors (Siegmond & Biermann, 1992; Steinberg et al., 1995; Grillitsch et al., 1999).

6.4 Digression: Biomonitoring

Some of the research done within the scope of this study may potentially result in an enhancement of recently used biomonitoring methods. Although biomonitoring was not the subject of this study, behaviour was used as a biomarker for indicating the presence of a stressor and that is the aim of biomonitoring, too.

Automated biomonitoring or biological early warning systems are defined as systems that detect toxic conditions on a continuous basis in whole organisms (Butterworth et al., 2000). In contrast to physico-chemical analyses biomonitoring facilitate an unspecific indication of pollutants including synergistical and antagonisti-

cal effects, in water monitoring. Using behavioural endpoints for biomonitoring has the advantages a) that their high sensitivity is comparable with other toxicological tests, e.g., enzymatic tests (see 6.1), b) that they have the capability of an online monitoring process without disturbing the test organisms and c) that organisms respond with behavioural changes within short time periods (Blübaum-Gronau et al., 2000).

Quantifying fish behaviour in the present study by using the online video-processing system BehavioQuant[®] (Spieser et al., 2000) proved to be useful for the detection of discontinuities and slight alterations in the normal behaviour. The experimental design of this study was developed under the explicit heeding of different standards (see 4.2 and Baganz et al., 2000). The tests organisms were sufficiently acclimated to the standard test conditions to enhance the reliability of the measurement by reducing higher variability of behaviour; recommended are 2-4 weeks (Siegmond and Biermann, 1990; Baganz et al., 2000). Even a sound experimental design combined with proper statistical analyses is essential for approaches using fish movement for the bioindication of stressors (Vogl et al., 1999).

The present study showed irrefutably that in behavioural experiments different distinctive patterns of animals' reactions need to be necessarily considered, depending on the species, the stressor and the specific time of the day. Since critical concentrations at a very low level can be measured and potential dose-effect relationships can be registered, biomonitoring using fish behaviour is about to become a standard. On this way some research has to be done to simplify the modelling of answer reactions detection to make it more reliable and more independent of the kind of reaction. So the results of this study indicate that in some cases it is necessary to register the absolute deviation from standard values independent on the direction of the reaction (e.g., if there is an increase or a decrease of activity and/or of the amount of rhythmical parameter). Furthermore for a successful use of behavioural tests in biomonitoring circadian periodic changes of activity should be regarded to prevent false alarms. That means that the alarm algorithms of biomonitoring should be calculated by defining a range of valid values following the temporal pattern of swimming behaviour under standard test conditions.

Some current biomonitoring systems use constant light conditions (LL) to reduce the circadian deviations of activity by eliminating the external zeitgeber. However

it is probable that this procedure generates a stress potential for the test organisms. In addition the absence of zeitgeber reduces the synchronisation between all single fish of the school and may lead therefore to a higher variability of the behavioural pattern which is not useful in the context of biomonitoring.

The experiments of the present study were performed over rather long time intervals and so the focus was on circadian rhythms. During the shorter time observations used for biomonitoring and pre-warning especially ultradian rhythms have to be taken into account, because they can indicate disturbances even in time periods in the range of minutes. Ultradian rhythms (which have a period length of oscillation smaller than 20 hours; $\tau < 20$ h) are commonly based on oscillations at the subcellular, cellular or supra-cellular level (Peters and Veeneklaas, 1992). The procedures of time series analysis that proved to be useful for analysis of circadian rhythm in this study can easily be exploited for the analysis of ultradian rhythms in biomonitoring applications.

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8. Appendix

8.1 Glossary

Acrophase	Phase angle corresponding to the maximal value of the rhythmic parameter studied
Circadian	Period of oscillation is 24 ± 4 hours
Cosinor-analysis	Nonlinear regression procedure (applying a parameterised cosinus equation to the raw data series)
Degree of Functional Coupling	Percentage of the circadian component and harmonic ultradian components in relation to all significant rhythmic components of a spectrum based on the respective relative parts of their assigned variance
Harmonic periods	Period lengths are integer dividers of 24 h
Harmonic portion	Percentage of the number of the circadian and their harmonic ultradian components in relation to the number of all significant periods smaller or equal to the circadian rhythm
Infradian	Period length of oscillation is more than 28 hours
MESOR	Mean of oscillation of cosinor analysis
Motility	Swimming velocity in video-pixels per second
Number of turns	Number of changes of the direction per second
Phase-shift	Displacement of an oscillation along the time axis
Polar Plots	Graphical presentation of amplitude and acrophase
Power spectral analysis	Fourier transformed autocorrelation function for quantification of the harmonic frequency structure of activity rhythms
Ultradian	Period length of oscillation is less than 20 hours
Zeitgeber (time trigger)	Forcing oscillation which entrains a biological rhythm

8.2 Abbreviations

MC-LR	Microcystin-LR
PCB 28	2,4,4'-trichlorobiphenyl (C ₁₂ H ₇ Cl ₃)
PCB	Polychlorinated biphenyl
LOEC	Lowest observed effect concentration
LC ₅₀	Lethal Concentration 50. It is the concentration of a chemical which kills 50% of a sample population.
LD ₅₀	Lethal Dose 50. It is the dose of a chemical which kills 50% of a sample population.
log K _{ow}	log of a compound's octanol/water partitioning coefficient
DFC	Degree of Functional Coupling
AhR	Aryl hydrocarbon receptor
MW	Molecular weight
ChE	Cholinesterase
τ	Period of biological rhythm
FDA	US Food and Drug Administration

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8.3.1 Figures

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8.4 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung.

Weder bin ich im Beseitz eines entsprechenden Doktorgrades noch habe ich mich an anderer Stelle um die Erlangung eines entsprechenden Doktorgrades beworben.

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