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Testing spatial avoidance and behavioural changes in European seabass in a floating pen in response to sounds from the FaunaGuard-Fish Module (FG-FM)

Research report 1

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Content

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Executive summary

Background

Aquatic animals live in a world with limited opportunities for vision, while water is an excellent medium for sound propagation. Most of them are also well capable of hearing. Consequently, many marine mammals and fishes make sounds for communication and use sounds from their environment during activities that are critical to survival and reproduction. The acoustic nature of the underwater world and the widespread auditory sensitivity also make aquatic animals vulnerable to noisy human activities that can cause temporary or permanent hearing loss, mask, disturb and deter. However, underwater acoustics also provide opportunities for monitoring and management.

GEMINI has been conducting construction activities in the Dutch North Sea for building an offshore wind farm. These construction activities include pile driving activities that will yield sound exposure at levels that are potentially harmful to nearby fishes and may have negative effects on local individuals and the local fish community for a certain amount of time. It is in the context of these activities and threats that Van Oord Dredging and Marine Contractors has been exploring whether sound can also protect fishes and whether sound playback could be an effective mitigation measure against over-exposure.

SEAMARCO and van Oord have developed an Acoustic Deterrent Device (ADD), equipped with a sound sequence specifically for fish, labelled the FaunaGuard Fish Module (FG-FM). The ADD is aimed at deterring fish out of an area that will be exposed to extreme sound levels that may induce serious harassment or harm (such as explosives or pile driving). The FG-FM has been applied in the field in Sweden, Brasil and the Netherlands and anecdotal evidence suggests that it works well and could potentially save a lot of fish.

So far, the FG-FM sound stimuli have only been tested in a fish tank context, in which the potential to trigger a startle response was used to improve the deterrent capacity of the 20 sounds that are put into a sequence with variable interval duration. However, a startle response does not necessarily relate to a tendency to show avoidance behaviour and the sound field of a fish tank is different from the outside conditions of open water. Furthermore, the limited space available to flee or the solid nature of the enclosure may restrict natural responses of spatial avoidance.

Objective and methods

The objective of the current study was, therefore, to test the behavioural effect of exposure to the FG-FM sound series in more natural sound field conditions with fish in floating pen that are less restricted in their swimming patterns to allow exploration of a more natural spatial response. We conducted this study with a replicated set of 16 groups of four individual seabass (*Dicentrarchus labrax*), swimming around in a large floating net pen (Ø 11.5-12.5 m) in a sheltered harbour of at least 3m depth during the experimental trials. The origin of the fish was a hatchery and they were tracked in 3D by telemetry.

In previous experiments, using the very same set-up, exposure tools and fish species, typical response patterns were diving down the water column, changing swimming speed and group coherence. The first of these parameters has been the most consistent in both indoor and outdoor conditions. In addition to these three parameters, we also measured the 3D-distance towards the speaker to test for spatial avoidance (taking distance in the horizontal plane into account as well as depth). We assessed the four behavioural parameters before, during and after the sound exposure and explored the detailed tracking for behavioural response correlations with sound level and individual sound stimuli.

Application of the FG-FM will induce a sound level gradient with the highest levels close to the device (and presumable close to the danger area) and fading sound levels with distance depending on local propagation properties. In order to explore the effect of the FG-FM sounds at the range of sound levels encountered in the field, and with a dose-response curve as explicit target, we tested behavioural responses to the maximum output level and a step-wise series of lower amplitude. We also tested broad-band white noise sounds in the same temporal pattern as the FG-FM sounds at a slightly lower but similar amplitude level as a control.

Results and interpretation

The sound exposure treatments and animal tracking tools have all been successfully executed according to the research design and yielded a replicated series of detailed records on the behaviour of 16 groups of four fish, responding to 48 trials of FaunaGuard sounds and 48 trials of white noise sounds of varying sound level (each group was exposed to six trials in two days). Unexpectedly, there were no consistent response patterns to sound exposure in any of the four parameters: there was no significant decrease in swimming depth, no significant change in distance to the speaker, and also no significant change in swimming speed or group coherence.

Detailed exploration of individual response patterns did reveal several sudden changes in behaviour associated with the onset of sound exposure for both the FaunaGuard and the control sounds, especially for the trials with higher sound levels. Furthermore, a quantitative comparison of distance to the sound source revealed quite a few groups that approached the

FaunaGuard sounds in the trial with the six highest sound levels. However, overall, there was no significant effect of the FG-FM on behaviour and sound level did not explain a statistically significant amount of the variation. We were therefore unable to generate a dose response curve.

Given that previous experiments with different sound stimuli have triggered consistent response patterns at high, but also at very low levels, we argue that the FG-FM sounds (and the white noise control sounds) were less suitable to elicit significant changes in behaviour than those sounds of previous experiments in the current test conditions and set-up and need higher sound levels to potentially trigger the same behavioural effects. There are a number of possible explanations for this result. 1) The batch of fish is less sensitive than previous batches, which were of the same species and of similar size, but came from another hatchery. 2) The temporal pattern of sound stimuli in earlier experiments (relatively brief pulses and brief intervals) were more potent in triggering a response. 3) The tones and spectral composition of FG-FM sounds and the white noise control sounds were spectrally less salient than previously used sounds (brown noise).

Conclusion and recommendations

There is no evidence that seabass would be affected in their behaviour, or would move away from the sound source, in an area surrounding the FaunaGuard in which the sounds are audible but not above the maximum currently tested. It could still be that more close to the FaunaGuard, or at sound levels above the maximum currently tested, the FG-FM sound series may have a spatially deterrent effect on seabass or make them move down the water column. Furthermore, given the anecdotal evidence from efficacy of the FG-FM during field applications, it could be possible that 1) free-ranging fish respond differently and stronger than captive fish in a net pen; and that 2) fishes from other species or background than hatchery-reared seabass respond differently and stronger. Future testing in free-ranging fish or with fishes of different species and background in a net pen is needed to exclude or confirm these uncertainties.

The current findings and review do suggest that it is useful to further explore acoustic response tendencies of fish and that it is likely still possible to improve the deterrence capacity of the FG-FM sound stimuli, given that: a) previous experiments in the very same settings did trigger consistent responses at much lower sound levels (for seabass swimming down, not swimming away), b) that tones have been reported in the literature to be suitable for conditioning, but to be much less efficient in eliciting a spatial avoidance response than a complex, broad-band sound, c) that successful applications of acoustic fish guiding, as reported in the literature, often use brief and broadband sounds with relatively short intervals, and d) that we know still very little about the potential effect of using multiple sounds in a sequence. We therefore recommend the exploration of effectiveness in triggering acoustic responsiveness and we suggested several explicit directions in terms of temporal and spectral sound stimulus features.

We believe the most logical next steps for concrete tests would be:

1. Test same seabass batch to sounds of different temporal and spectral pattern in a net pen (like Neo et al. 2016) – done, data will be processed for Research report 2.
2. Repeat test of FaunaGuard sounds with other batch of seabass and add a more pelagic fish species – potential experiment for the net pen in the future.
3. Compare responsiveness to FG-FM sounds directly to pulse train like in Neo et al. (2016) at different sound levels to assess dose-response curve – can be combined with 2.
4. Investigate the effect of alternating or varying sounds in a sequence on response tendency and habituation – long-term plans, adequate acoustic contrast tests should be included.
5. Apply the FG-FM sound exposure at two distinct field sites with virtual source location of anthropogenic acoustic danger and monitor free-ranging fish by telemetry – critical for final evaluation of efficacy.
6. Assess source level of FaunaGuard and model spatial soundscape gradient for areas of application – not an explicit target of the current project and objectives and TNO-expertise.

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1. Introduction

Many aquatic and marine animals are affected by and potentially flee from human activities and the associated anthropogenic sounds. The interruption of activities and spatial avoidance of an area used for foraging, resting or spawning may be detrimental to individual animals and species (Richardson et al. 1995; Nowacek et al. 2007; Clark et al. 2009; Radford et al. 2014; Kunc et al. 2016). However, fleeing from anthropogenic sounds may also be adaptive as there are human activities and tools that are much more harmful to wildlife than the potential consequences of interruption and avoidance. Water inlets for example at hydro-, tidal or nuclear power plants or other pumping stations may cause direct physical damage and death to entrained fish (Schilt 2007; Keefer et al. 2013; Pracheil et al. 2016). Also loud sounds from pile driving, seismic surveys and explosions may cause hearing deficiencies, injury or death to relatively nearby animals (Popper & Hastings 2009; Slabbekoorn et al. 2010; von Benda-Beckmann et al. 2015). Finally, marine mammals attracted to aquaculture or fishing nets may not only affect the harvest or catch rate negatively, but may also get entangled and caught (Read 2008; Reeves et al. 2013; Peltier et al. 2016). Consequently, deliberate acoustic deterrence of aquatic animals has management potential for the protection of marine predators such as dolphins and seals, but also for fishes and fisheries (Bomford & Obrien 1990; Jefferson & Curry 1996; Popper & Carlson 1998; Shakner & Blumstein 2013; Götz & Janik 2013; 2015).

1.1 Acoustic deterrence experience in marine mammals

Most experience with acoustic deterrence is in the context of marine mammals and bycatch and depredation problems in fisheries (e.g. Anderson & Hawkins 1978; Kraus et al. 1997; Olesiuk et al. 2002; Fjalling et al. 2006; Gazo et al. 2008; Read 2008; Waples et al. 2013; Mangel et al. 2013; Reeves et al. 2013). Acoustic deterrent or harassment devices (ADDs and AHDs) produce loud sounds to keep fish predators such as seals and dolphins away from aquaculture and fishing nets or away from potentially harmful human activities, such as explosions or pile driving. The application may yield immediate alleviation of the problems, although habituation may limit long-term efficacy (Cox et al. 2001; Rankin et al. 2009). Furthermore, there are also concerns about unwanted side effects such as hearing loss in target species and possible impact on other wildlife (Dawson et al. 1998; Brandt et al. 2013). Furthermore, the ADD may even become a sort of ‘dinner bell’ when animals learn that the sound is not associated with any danger but with an exceptional aggregation of food (Carretta & Barlow 2011; Shakner & Blumstein 2013). This problem does not apply to keeping animals away from detrimental impact areas without any reward.

Specific for fisheries applications, so-called ‘pingers’ have been developed: a subset of ADDs which generate high-frequency sounds at relatively moderate levels to induce local aversion to keep marine predators away from cages or nets (Kastelein et al. 1997; Dawson et al. 1998). Several field studies report on successful use of acoustic alarm signals from pingers to keep dolphins and whales away from human fishing activities and reduce interactions with fishing nets (Bordino et al. 2002; Barlow & Cameron 2003; Carretta et al. 2008; Waples et al. 2013; Mangel et al. 2013; Cruz et al. 2014; Larsen & Eigaard 2014). A nice example is reported by Culik et al. (2001), in which harbor porpoise were successfully kept away from a fishing area by pinger use (see Fig. 1), while catch rates of herring (*Clupea harengus*) remain unaffected by the sounds from this device. Studies on captive animals have provided complementary insight into acoustic efficacy to induce startle or spatial avoidance responses and explored the potential to avoid habituation by variation in structure and timing of stimulus presentation (e.g. Teilmann et al. 2006; Kastelein et al. 2006; 2014; Götz & Janik 2011; and see Shapiro et al. 2009).

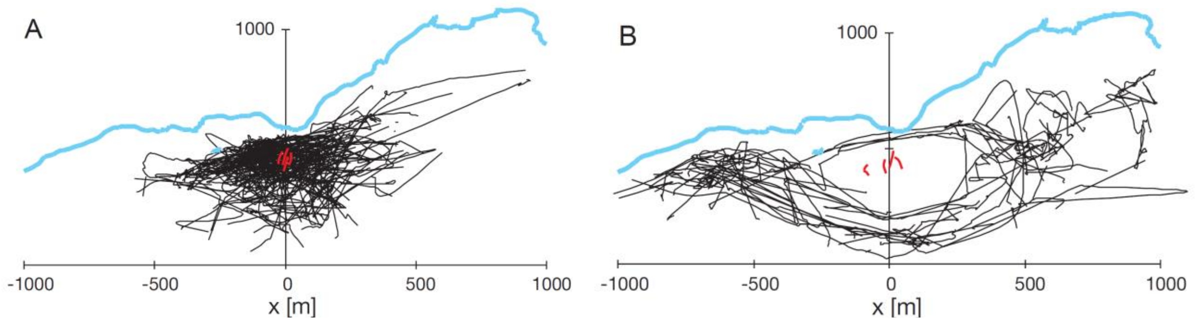


Figure 1: Example of an effective demonstration of the use of pingers to keep harbour porpoise (*Phoecoena phoecoena*) away from fishing nets at Fortune Channel, Clayoquot Sound, Vancouver Island, Canada. The blue lines represent the coast line. The red are tidal float-lines for nets with pingers and the black lines are swimming tracks of porpoise groups observed from the coast by theodolite. A: Situation before pingers are turned on; B: Situation during the acoustic alarm made by the pingers (Culik et al. 2001).

1.2 Fish hearing and deterrence efforts

Hearing sensitivity and functionality is also widespread among fishes (Tavolga 1971; Hawkins & Chapman 1976; Popper & Fay 1993; 2011). Most fish species rely on hearing for daily activities that are critical for survival and reproduction (Myrberg 1981; Slabbekoorn et al. 2010; Amorim et al. 2015). They can communicate with sounds for mate attraction, territorial disputes and social or reproductive aggregations. They can also find prey via acoustic cues, detect looming predators by sound, or use the soundscape for orientation and navigation (Slabbekoorn & Bouton 2008; Fay 2009). Acoustic detection abilities vary among taxa and age classes in absolute levels and spectral range. Depending on species-specific hearing adaptations and the presence of a swim bladder, fish sense the particle motion and sound pressure components of sound (Zeddies et al. 2012; Nedelec et al. 2016). Species without hearing specializations or swim bladder are the least sensitive and typically only hear up to about 300 Hz. Many fish species hear up to 1000 Hz, while the most specialized species can hear up to 4000 Hz. There are some exceptional species that even hear into the ultrasonic (Popper et al. 2004). Given the variable but widespread hearing abilities, it should be no surprise that there are also effects of anthropogenic sounds on fishes in terms of startle responses (Blaxter et al. 1981; Kastelein et al. 2008; Wardle et al. 2001) and a variety of escape swimming patterns (Nelson & Johnson 1972; Sarà et al. 2007; De Robertis & Handegard 2013; Hawkins et al. 2014).

The spatial responsiveness of fish to anthropogenic sounds has stimulated acoustic fish guiding efforts especially in the context of riverine passage problems for migratory fish (Haymes & Patrick 1986; Knudsen et al. 1993; 1997; Popper & Carlson 1998; Schilt 2007; Noatch & Suski 2012). There are several success stories with sounds of relatively extreme frequencies, both in the ultrasonic and the infrasonic ranges (above (> 20.000Hz) and below (< 20Hz) the human hearing range, respectively). Gibson & Meyers (2002) reported ultrasonic deterrence and significant reductions of American shad (*Alosa sapidissima*) and alewife (*Alosa pseudoharengus*) caught in the turbine tube of the Annapolis Tidal Hydroelectric Generating Station, Nova Scotia, Canada. Gurshin et al. (2014) reported on the history and most recent improvements for another successful ultrasonic fish guiding project on blueback herring (*Alosa aestivalis*) at the Crescent hydropower dam in the Mohawk river, New York, USA. In Europe, several studies on fish guiding efforts have a bias towards the use of low-frequency sounds. Sand et al. (2000) showed that the spatial distribution of passing European eel (*Anguilla anguilla*) could be shifted from one river bank towards the other by emitting underwater infrasound. Sonny et al. (2006) applied the same infrasound source to divert fish away from central target corridors at the cooling water inlet of the nuclear power plant, at Tihange, Belgium.

There are also applications of acoustic deterrence with sounds of less extreme frequencies that should be audible to a wide variety of fishes. Maes et al. (2004) studied effects of a source that generated sounds in the range of 20-600Hz and reported large reductions in trapped herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) at the cooling water inlet of the nuclear power plant at Doel, Belgium. Sound has also been applied in combination with strobe lights or bubble screens, which proved to be successful at relatively low frequencies (5-600Hz) in guiding juvenile Chinook salmon (*Oncorhynchus tshawytscha*) away from a low-survival migration route (Perry et al. 2014) and at a higher frequency range (500-2000Hz) in curbing the upstream migration of invasive silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) (Ruebush et al. 2012). However, the spread of successful applications, and long-term efficacy in particular, may be more limited than appears from these reports. Publication effort may be biased to positive results and there are still few independent scientific studies available in the peer-reviewed literature.

1.3 Exploring responsiveness to acoustic stimulus features

Indoor studies in large tanks and outdoor studies in ponds or floating pens have been used to explore effects of sound on fish behavior, which may provide insights into ADD efficacy and potential for improvement (e.g. Kastelein et al. 2007; 2008; Racca et al. 2014; Neo et al.

2014; 2015; 2016). Taylor et al. (2005), for example, used a random series of cyclic sound bursts at frequencies ranging from 20-2000Hz (details on temporal pattern not provided) to raise the efficacy of a bubble curtain as a behavioural barrier in an indoor test with bighead carp. Duning & Ross (2010) assessed habituation tendency reflected in reduced response intensity to repetitive ultrasonic sound. Sand et al. (2000) found little evidence for habituation to infrasound bursts lasting 20 s at 3–5 min intervals in 12–15 successive stimulations on two consecutive days. Vetter et al. (2015) revealed that a complex, broad-band sound of a boat engine recording (0-10 kHz) is more potent in initiating startle and avoidance responses in silver carp than single-frequency tones (see Fig. 2). They showed that tones of 500Hz, 1000Hz, 1500Hz and 2000Hz triggered no responses, while the boat engine sound did. Playing back this sound at alternating sides of the outdoor pond made a group of fish redirect away from the sound source repeatedly up to 37 consecutive times.

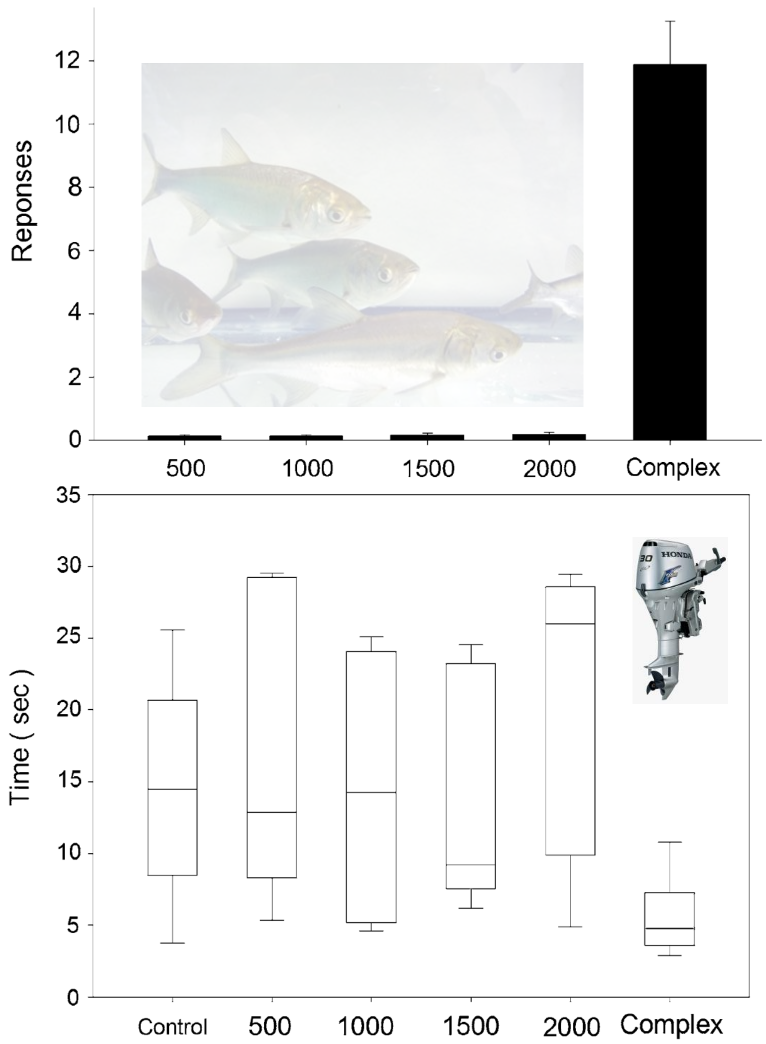


Figure 2: Response patterns of silver carp (*Hypophthalmichthys molitrix*) to experimental sound exposure in captivity (Vetter et al. 2015). Pure tones of various frequencies (500Hz; 1000Hz; 1500Hz; 2000Hz) trigger no avoidance response, while broad-band boat engine sound always triggered avoidance (top panel) and made the swimming speed go up, which is shown in the reduction of time needed to swim the first 2 meters after the start of the stimulus presentation (bottom panel) (see Fig. 23 for spectral distribution of energy of the sound stimuli associated with these data).

Neo et al. (2014; 2015) investigated behavioural effects of broad-band sounds on European seabass in a large fish tank at SEAMARCO, followed by studies in an outdoor floating pen at the Jacobahaven, Kamperland, Zeeland, the Netherlands (Neo et al. 2016; Neo 2016). Upon sound exposure, the groups of four fish typically startled, increased their swimming speed, swimming depth and group cohesion. Within the 30 or 60 min exposure trials, the fish behaviour returned back to baseline levels. This recovery was shown to be habituation instead of sensory adaptation or motor fatigue, as the fish could still respond to novel acoustic stimuli (Neo et al. 2015). This standard essay was practical for replication and suitable for testing effects of temporal structure on behavioural effects: impulsive sound had longer lasting effects than continuous sound (Neo et al. 2014) and pulse repetition interval had subtle but significant effects on post-exposure behaviour (Neo et al. 2015). In the floating pen setting, the effect of sound intermittency was similar but not as clear as in the fish tank (Neo et al. 2016). A 'ramp-up' procedure that gradually increased amplitude from the ambient level to the standard exposure level over 20 min. caused the fish to change behaviour in the same way as when they were exposed to sound treatment directly without a 'ramp-up'. However, the fish did not swim away from the sound source as expected. Moreover, some groups seemed to be attracted to the low-level sounds and seemed to habituate to the sound more quickly (Neo et al. 2016).

The outcome of the ramp-up procedure testing on seabass in the floating pen (Neo et al. 2016) calls for caution in applying sound deterrence in general. As sound attenuates over distance with underwater propagation (Shapiro et al. 2009), there will always be conditions of low and high sound levels. Consequently, it is important to realize that sound is not only a potential deterrent, but may also attract fish. Abiotic or artificial sounds, within the audible range for fish, may deter or attract depending on the level and temporal pattern of sounds (Nelson & Johnson 1972; Febrina et al. 2015). For example, loud and artificially regular sounds may be deterrent, while soft and naturally irregular may be more an attractant. The latter acoustic features may be perceived as generated by potential prey or a turbidity inducing event that will open up prey availability (Holt & Johnston 2011). Sloan et al. (2013) also showed that common carp (*Cyprinus carpio*) could be conditioned to respond phonotactically within three days by associating a food reward with a 400 Hz tone (30 second presentation, two seconds of sound alternated with one second of silence).This is in line with Willis et al. (2002), who showed associated learning with 600Hz, 800Hz and 1000Hz tones, as well as an apparently inherent phonotactic response to feeding sounds in grass carp (*Ctenopharyngodon idella*).This indicates that there is ample flexibility in spatial responses to sounds, likely driven by natural punishment and reward, but that phonotactic behaviour is part of the natural behavioural repertoire of fishes (also see Febrina et al. 2015; Moynan et al. 2016).

Spatial deterrence may also depend on the space available for test fish to swim (Kastelein et al. 2011; Neo et al. 2016) and ADDs should always be tested *in situ* to confirm operational qualities under field conditions (Jacobs & Terhune 2002; Slabbekoorn 2016). However, the study by Vetter et al. (2015) suggested that broadband sounds in combination with an inconsistent amplitude may be more effective deterrents and studies by Neo et al. (2014;

2015; 2016) also suggest that relatively brief broad-band pulses generated with brief intervals may be effective deterrents. The application of ultrasound in the deterrence studies on the American *Alosa* species may support this assumption as ultrasonic sound patterns concerned band-passed white noise with most energy between 122 and 128 kHz in so-called 33% duty cycles, with pulses of 0.5 sec and inter-pulse intervals of 1.0 sec (Gibson & Meyers 2002; Gurshin et al. 2014). Also Maes et al. (2004) used relatively broad-band, band-passed white noise bursts, this time with most energy between 20–600 Hz, repeated every 0.2 sec. However, the *in situ* infrasound studies, showing effective deterrence for a variety of species, including European eel, herring and sprat, concerned different and variable exposure regimes: a 12 Hz sound, presumably emitted continuously (Sand et al. 2000) and a 16 Hz sound, emitted in 30 sec periods, repeated every 2-4 min (Sonny et al. 2006). In general, there are no explicit tests of the role of temporal patterns in the efficacy of ADD's.

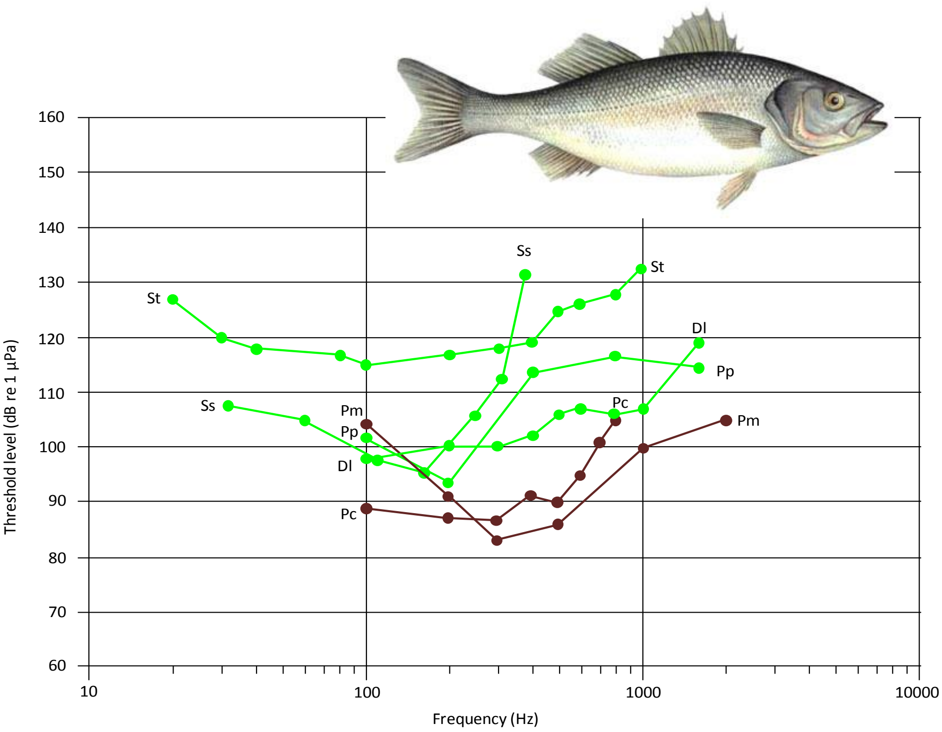


Figure 3: Hearing curves for European seabass and other benthopelagic fish species: Dl = *Dicentrarchus labrax* (seabass, Fam. Moronidae). Seabass are most sensitive in the range of 100-400 Hz and also hear up to 1000 Hz, but become rapidly insensitive to soud at higher frequencies. Ss= *Salmo salar* (salmon, Fam. Salmonidae); St= *Salmo trutta* (sea trout, Fam. Salmonidae); Pp= *Pungitius pungitius* (nine-spined stickleback, Fam. Gasterosteidae); Pm= *Pagrus major* (red seabream, Fam. Sparidae); Pc= *Pogonias chromis* (black drum, Fam. Sciaenidae). Green = species of the North Sea. Brown = species of the same family as a species of the North Sea area (from Bouton et al. SONIC-report 2015).

1.4 The FaunaGuard

Kastelein et al. (2007; 2008) tested startle response tendencies in a fish tank to a variety of sounds in different North Sea fish species: seven marine mammal pingers in five species

(Kastelein et al. 2007) and single frequency tones across the relevant range for fish and beyond in eight species (Kastelein et al. 2008). The data showed startle response patterns that were variable among species and restricted to relatively low frequency ranges, matching fish hearing ranges (Fig. 3), but not necessarily matching the shape of their hearing curves. In 2010, SEAMARCO and van Oord Dredging and Marine Contractors collaborated on the development of an ADD, called the Universal Fauna Guard (UFG), and designed a separate sound sequence for fish, labelled the Fish Module (FM) The UFG consists of a sound generator, a power amplifier, and an underwater loudspeaker (Kastelein et al. 2011; Van der Meij et al. 2015). The UFG prototype (UFG-01) was used at a dredging site in Sweden for 6 months and Kastelein et al. (2011) reported: “very few fish died as a result of underwater explosives (when the UFG was activated), but a large number died when, on rare occasions, the UFG was not activated before an underwater explosion occurred”.

The UFG-01 consisted of a series of twenty 10-second tones with variable intervals, for which the responsiveness of individual sound stimuli was tested at variable sound levels in two fish species by another series of startle response tests to adjust the ADD and improve its deterrence efficacy (Kastelein et al. 2011). Eight groups of the two fish species were used for these tests: five groups of three size classes of sea bass (*Dicentrarchus labrax*) and three groups of one size class of the thicklip mullet (*Chelon labrosus*). Half of all sounds from the now labelled FaunaGuard FishModule (FG-FM) resulted in a distinct startle response. Especially all sounds played at relatively low amplitude (around 150 dB re 1 μPa) or with most energy above 700 Hz triggered fewer startles. An effect of wave type on response tendency was not reported (and maybe also not to be expected when wave type is determined by harmonic components that are beyond the hearing range of the fish). There were no obvious differences between the species or among the size classes (response tendencies for the groups of small and large seabass were typically within the range of variation among the three different seabass groups of the same intermediate size).

The sounds that did not yield a strong response in Kastelein et al. (2011) were replaced in the latest FG-FM stimulus series, which now consists of ten tones of different frequencies and wave types followed by a variable set of upsweeps, downsweeps and more complex frequency-modulated sound elements and a noise band (see Table 1). Another pilot exposure test was done at a single fish community tank (Sea aquarium “het Arsenaal”) with a wide variety of different fish species present, all exposed at the same time as a group to unknown sound levels (Kastelein et al. 2012). The rationale behind having a variable series of sounds, with each subsequent one being acoustically distinct, was to counteract habituation (c.f. Teilmann et al. 2006; Rankin et al. 2009; Neo et al. 2015; Radford et al. 2016).

1.5 Remaining questions and current objectives

It is currently unclear whether the startle capacity of the FG-FM sound series as reported for two species in a fish tank can be extrapolated to outdoor conditions in open water with more natural sound field conditions (c.f. Neo et al. 2016; Neo 2016). It is unknown whether the FG-FM sounds would trigger a vertical downward shift in the water column or a horizontal avoidance response. Furthermore, we have no dose-response data for any behavioural change yet and we also do not know whether low sound levels could potentially attract fish. The aim of the current study was to fill in these gaps in our knowledge by testing behavioural responsiveness of groups of four seabass individuals as a model system in a floating pen in outdoor conditions with the following objectives:

1. Determine whether the fish show behavioural changes that may indicate anxiety (diving down, speeding and clustering together) during exposure to the FG-FM-sounds in a natural sound field.
2. Determine whether the fish show spatial avoidance in the horizontal and vertical plane (3D-distance to the speaker) during exposure to the FG-FM-sounds in a natural sound field.
3. Assess a dose response curve for spatial avoidance by exploring the range of sound levels at which there is no response up to a maximum response.
4. Explore whether there are sound levels at which the FG-FM elicits a phonotactic response with fish approaching rather than avoiding the speaker.

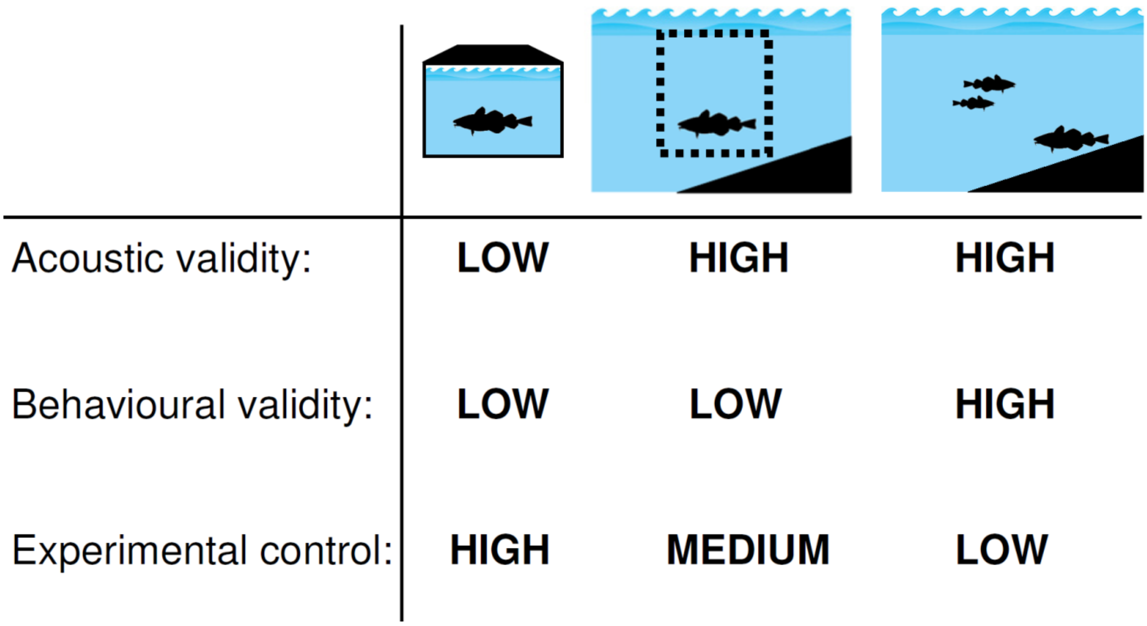


Figure 4: Evaluation of constraints and potential for different experimental settings for studies on behavioural responsiveness to sound in fish. The three settings evaluated concern: 1) indoor studies using fish tanks or moderately sized basins; 2) outdoor studies using captive fish that can move around in a restricted area; 3) outdoor studies on free-ranging fish that happen to be around at the selected study area. The settings can yield complementary data but vary in acoustic and behavioural validity and the potential to: control experimental design, replicate adequately, and take all necessary measurements (from Slabbekoorn 2016).

Note that test conditions in a fish tank and a floating pen yield complementary insights but still do not equal field conditions of free-ranging fish (Fig. 4). Startle responses can but do not necessarily relate to fleeing or diving patterns and fleeing or diving patterns can but do not necessarily relate to spatial avoidance in free-ranging conditions. However, sound field conditions in terms of both sound pressure and particle motion are less natural in artificial tanks than in outdoor conditions. Behavioural responsiveness tests in a floating pen are therefore especially useful to test relative importance of sound features in eliciting any reaction and to explore whether the reaction has a directional nature relative to the location of the sound source. Translation of such insights (based on adequate replication, but typically gathered for a particular model species) still requires testing the actual application to free-ranging fish of different species and exploring the presence and size of zones of spatial avoidance and potential zones of approach tendencies (Fig. 5).

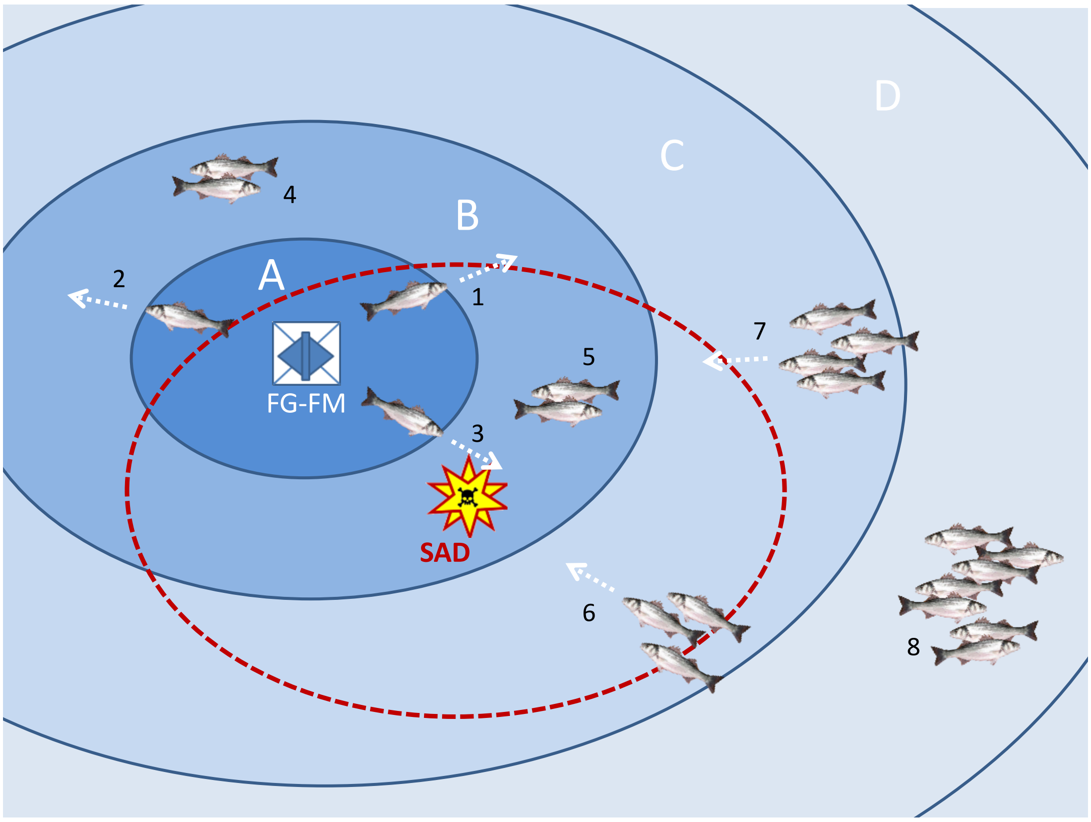


Figure 5: Schematic illustration of hypothetical zones around an Acoustic Deterrent Device (ADD), such as the FG-FM, placed at a certain distance from an anthropogenic Source of Acoustic Danger (SAD). The target is to deter fish away from and outside the area that may cause harm (red dashed oval), which is achieved for fish 1 and 2 that are in zone A in this example, but not for fish 3. Inherent to sound level decline with distance, beyond deterrent zone A, there may be a zone B, in which fish may startle or respond otherwise, but do not make a spatial shift, which yields no problem for fish group 4, but renders the ADD ineffective in saving fish group 5. Beyond zone B, there may be a zone C in which fish even exhibit phonotactic behaviour, approaching the ADD, inducing trouble for fish groups 6 and 7 (note that wider circles have a probability to involve a larger number of fish). In zone D, the ADD may still be audible but not having a spatial effect. The presence and size of each zone may depend on species and environmental conditions and will be determined by ADD sound features, source level and sound propagation (affected by e.g. depth, bathymetry, temperature and currents).

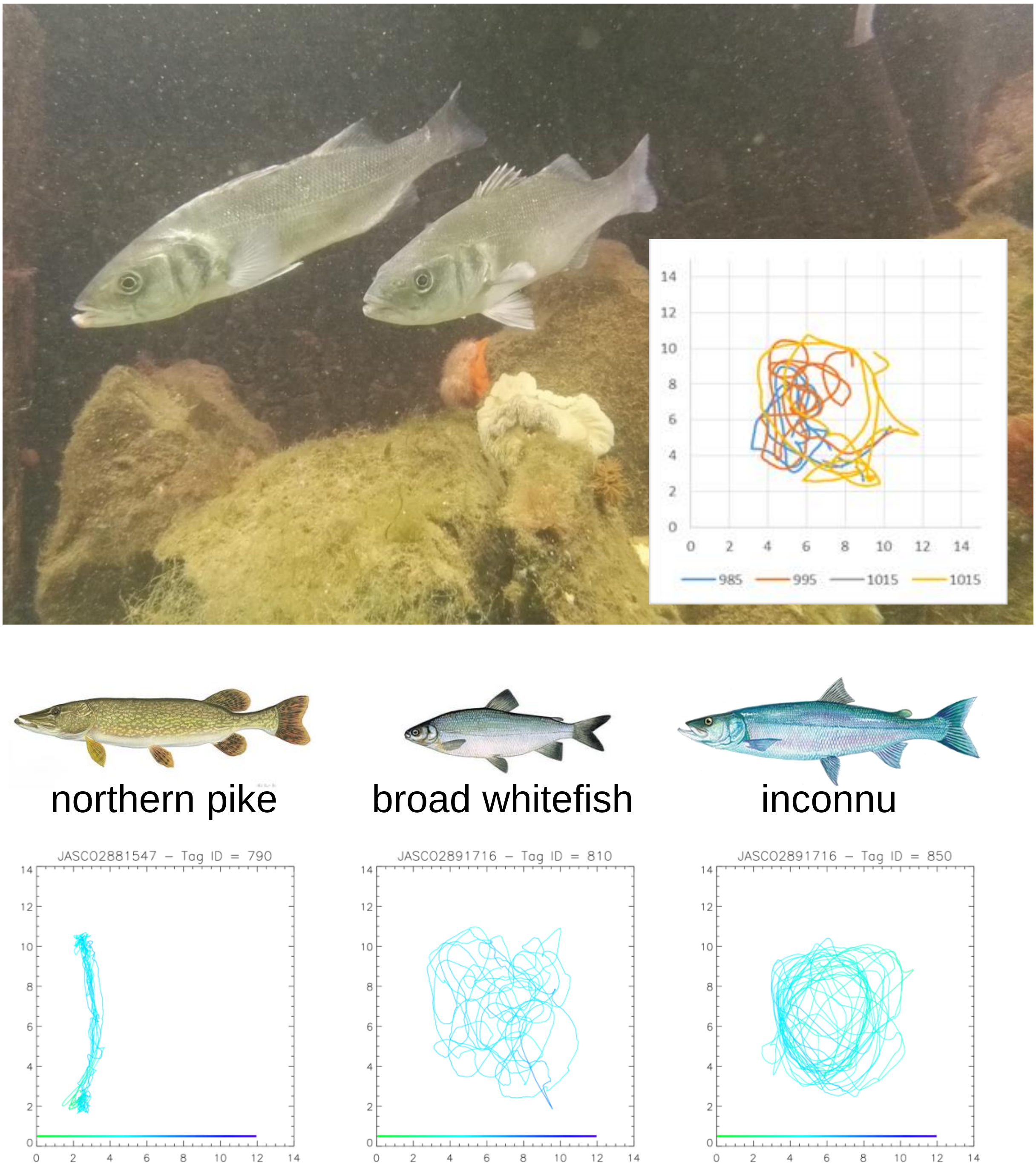


Figure 6: Two European seabass (*Dicentrarchus labrax*) individuals in a tank at Stichting de Zeeschelp (picture in upper part) with a typical swimming pattern of four individuals (each a different colour) in the floating pen in the Jacobahaven, as tracked by the HTI telemetry system. Such swimming patterns in captive conditions are species-specific and likely related to species-specific swimming in free-ranging conditions. In the bottom part, three examples of distinct swimming patterns in similar conditions of three other species (from Racca et al. 2004). The seabass are most similar to the inconnu (*Stenodus leuichthys*), which swims mostly in circles, and sometimes they are more like the broad whitefish (*Coregonus nasus*), which swims in more irregular patterns. They thereby provide a suitable model to test spatial avoidance and are unlike the northern pike (*Esox Lucius*), for which the assessment of behavioural effects on its more restricted swimming pattern may be more challenging.

2. Materials and methods

2.1 Test animals

We used 16 groups of four European seabass (*Dicentrarchus labrax*) of 35 to 40 cm in body length (Fig. 6). The fish were acquired from a hatchery (FRESH, Völklingen, Germany) and kept in two indoor holding tanks (Ø 3.5 m, depth 1.2 m) at Stichting Zeeschelp (Kamperland, The Netherlands) in a dark-light cycle similar to outside day-night cycle. The water in the holding tanks was continuously refreshed with seawater from the Oosterschelde, an estuary of the Dutch North Sea. The fish were fed with commercial pellets (Aller Blue Organic EX 8 mm, AllerAqua), for which the amount was determined by the temperature of the water. All experiments were conducted in accordance with the Dutch Experiments on Animals Act and approved by the Dutch Central Commission Animal Experiments (CCD) under no. AV D106002016610.

European seabass are a benthopelagic species that has been used in several studies aimed at exploration of acoustic sensitivity (Kastelein et al. 2011; 2012; Neo et al. 2014; 2015; 2016; Neo 2016; Radford et al. 2016). They have proven to be a suitable species in terms of behavioural resolution to variation in sound exposure regimes and are not expected to be an exceptionally sensitive or particularly robust species. The choice of origin has always been a hatchery as wild-caught fish are more difficult to get and will likely be a less homogenous group in for example size, age or experience compared to a hatchery batch. As addressed in the introduction, there are also limitations to working with a single model species, in captive conditions and from a hatchery background, but the choice is highly suitable for the current objectives.

2.2 Experimental arena

The experiment was conducted using a study island as has been used for previous sound exposure studies (Neo et al. 2016; Neo 2016), which was placed in the Jacobahaven, a man-made cove in the Oosterschelde, The Netherlands (Fig. 7). The Jacobahaven is about 200 m wide, 300 m long and depending on the tides 2-5 m deep. The Jacobahaven is situated near the Oosterscheldekering and no external boat traffic is allowed within 1 km of the Oosterscheldekering. The study island was located in the middle of the Jacobahaven and anchored with dead weights, a combination of chains and stretchable bungee ropes kept the island in place throughout the tides.

We assembled the study island using a modular floating system (Candock, Canada), it consisted of a working platform for the equipment and researchers and an octagonal net pen (Ø 11.5-12.5 m, >3 m deep) as arena for the fish. The two platforms were separated with a 0.5 m distance to avoid noise transmission from the working platform to the net pen. The working

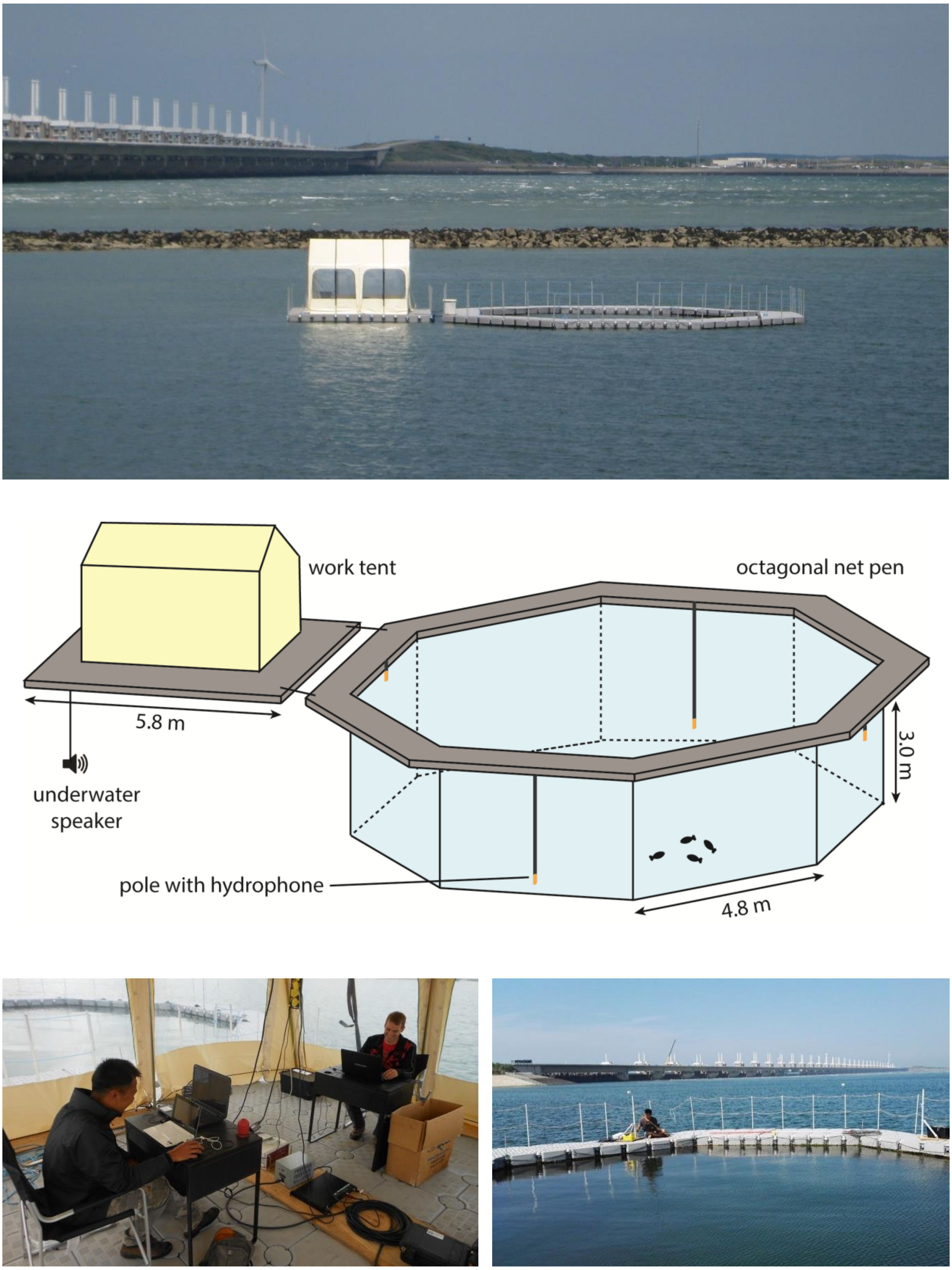


Figure 7: The floating pen and research platform in the Jacobahaven, Kamperland, Zeeland, the Netherlands. Landscape with Oosterscheldekering in the background at the top, schematic illustration with HTI hydrophone placement in the middle, and Errol Neo and Jeroen Hubert in the office work tent (left) and measuring the underwater soundscape (right).

platform supported the speaker, such that the distance from the speaker to the net was 7.8 m and unwanted near-field effects of the speaker were avoided. Detailed measurements of the underwater soundscape revealed a clear sound level gradient across the net pen (Fig. 8).

2.3 Tagging and tracking fish

We tracked the swimming patterns of the four fish in the net pen using acoustic tags (Model 795-LG, HTI, US) that emitted 307 kHz pings at a ~ 1s pulse rate interval (PRI). Fish could be identified and tracked individually because of tiny differences in the programmed PRI of the different tags. At the net pen, the pings of the tags were received by four hydrophones (Model 590-series, HTI, US), two close to the surface and two close to the bottom (Fig. 7), and stored on a laptop via a specialized oscilloscope (Model 291, HTI, US). The spatial resolution is determined by the potential of data on 60 fish locations per minute (for each of the four fish) and the amount of missing values for one or more hydrophones at any particular time point, due to signal collision or problematic signal-to-noise ratios. The percentage of missing value is typically around 20-30%, yielding spatial resolution of about 40 samples per minute.

Before tagging, the fish were anaesthetised in a bath with 2-phenylethanol (0.5ml/l seawater). Once anaesthetised, we moved the fish on its back in a holder, with its abdominal wall above water and its head submerged in seawater with half the amount of 2-phenylethanol (0.25ml/l) to maintain anaesthesia. The tag was implanted in the fish's abdominal cavity, therefore we made a small incision that was stitched after implantation (Fig. 9). After the tagging, the fish were kept in a rectangular tank (1.20 x 1.00 x 0.65 m) to recover.

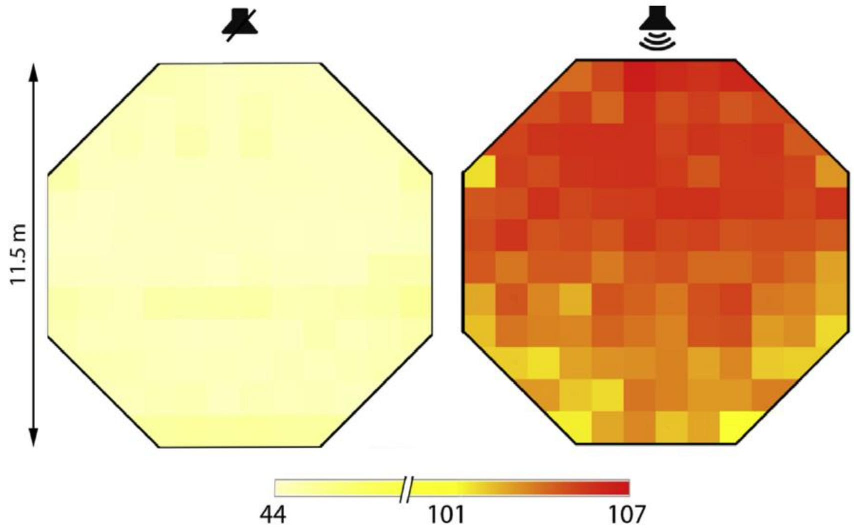


Figure 8: 2D soundscape maps for measurements taken for Neo et al. (2016) in sound velocity level (SVL, in dB re nm/s) prior to (ambient conditions) and during sound exposure, measured at 1.5 m water below the surface. The speaker was 7.8 m away from the experimental arena and fish were therefore always outside the postulated acoustic nearfield < 7.5 m (sound treatments had minimum frequency 200 Hz). The amplitude gradient was also clear for sound pressure level (not shown).

2.4 Treatment series

We exposed all groups of fish to three different sound exposures of the FaunaGuard-Fish Module (FG-FM) and three sound exposures of white noise as control (white noise concerns a random distribution of sound energy with equal intensity across frequencies). In previous experiments, we have used brown noise (also a random distribution of sound energy, but with a slight bias to be louder at lower frequencies), but white noise was chosen here to cover the frequency range of the FG-FM sounds without any spectral bias. We used the FG-FM sound treatment as provided: it consists of a repeated fixed sequence of 20 sounds of 10s with varying (3-10s) silence intervals between the sounds (Table 1). The control treatment was created in Audacity 2.0.5, we generated Gaussian white noise and created silences with the same interval and duration as in the FG-FM treatment.

To be able to create a dose response curve, we created 18 different relative amplitude levels of the original treatments. We created the different levels in steps of -3dB, ranging from -0 till -51 dB rel. We grouped the different levels into three categories: high (-0 up to -15 dB), medium (-18 up to -33 dB) and low (-36 up to -51 dB) amplitude. The recorded decrease in amplitude was ~ 2.4 dB re 1 µPa per step (Fig. 10).

Figure 9: Tagging holder and tools (left). Stitching the incision after tagging (right). The head of the fish is submerged in filtered seawater from the Oosterschelde during the tagging procedure, which takes 5-10 min.

The sounds were played back with an underwater transducer (LL-1424HP, Lubell Labs, Columbus, US) using a laptop, a power amplifier (DIGIT 3K6, SynQ) and a transformer (AC1424HP, Lubell Labs). To examine the actual sound levels present in the net pen, we measured sound pressure levels (SPL) and sound velocity levels (SVL) twice at six distances from the speaker (every 2.1 m, from 8.3 to 18.8 m from the speaker). The measurements were done using the M20 particle velocity sensor (GeoSpectrum Technologies, Canada), this sensor measures sound pressure using an omnidirectional hydrophone and 3D particle velocity using three accelerometers. Calibration of the sensor was provided by the manufacturer. The signals were stored on a laptop at 40kHz via a current-to-voltage convertor box (GeoSpectrum Technologies Inc., Canada) and a differential oscilloscope (PicoScope 3425, Pico

Technologies, UK). The recordings were later processed with the Matlab application paPAM (c.f. Nedelec et al., 2015; 2016) using a 200-1000 Hz bandpass filter.

2.5 Experimental design

We exposed each fish group to six different sound treatments: three FaunaGuard-Fish Module (FG-FM) exposures and three white noise (control) exposures. Both treatments are played back once at a high, medium and low amplitude (see 2.4). The relative levels of the FG-FM and white noise were the same for one group. The order of the treatments followed an incomplete counterbalanced design, 12 amplitude levels were played back three times and six levels were played back twice. Each group was exposed to three FG-FM treatments and three white noise treatments of the same levels, one from each category: high, medium and low (amplitude).

Table 1: Description of twenty 10-second sounds of the FaunaGuard-Fish Module (FG-FM) in the fixed order of appearance. Amplitude waves and sound levels can be found in Fig. 10. Amplitude waves and sonograms for the inspection and comparison of acoustic features can be found in Fig. 11, 12 and 13.

Order	Description
1	Square wave, F1 (fundamental frequency): 600Hz
2	Square wave, F1: 500Hz
3	Triangle wave, F1: 400Hz
4	Triangle wave, F1: 500Hz
5	Triangle wave, F1: 600Hz
6	Sawtooth wave, F1: 800Hz
7	Sawtooth wave, F1: 600Hz
8	Sine wave 300Hz
9	Sine wave 400Hz
10	Sine wave 500Hz
11	Up-sweep sine wave (0.5s) 100-600Hz
12	Up-sweep sine wave (0.5s) 200-700Hz
13	Up-sweep sine wave (0.5s) 200-1000Hz
14	Up-sweep square wave (0.5s) 200-1000Hz
15	Frequency modulation (0.5s) 900-250Hz
16	White noise 400-700Hz
17	Frequency modulation (0.5s) 1350-160Hz
18	Up-sweep (0.2s) 200-600Hz
19	Down-sweep (0.5s) 1000-200Hz
20	Down-sweep (0.5s) 600-200Hz

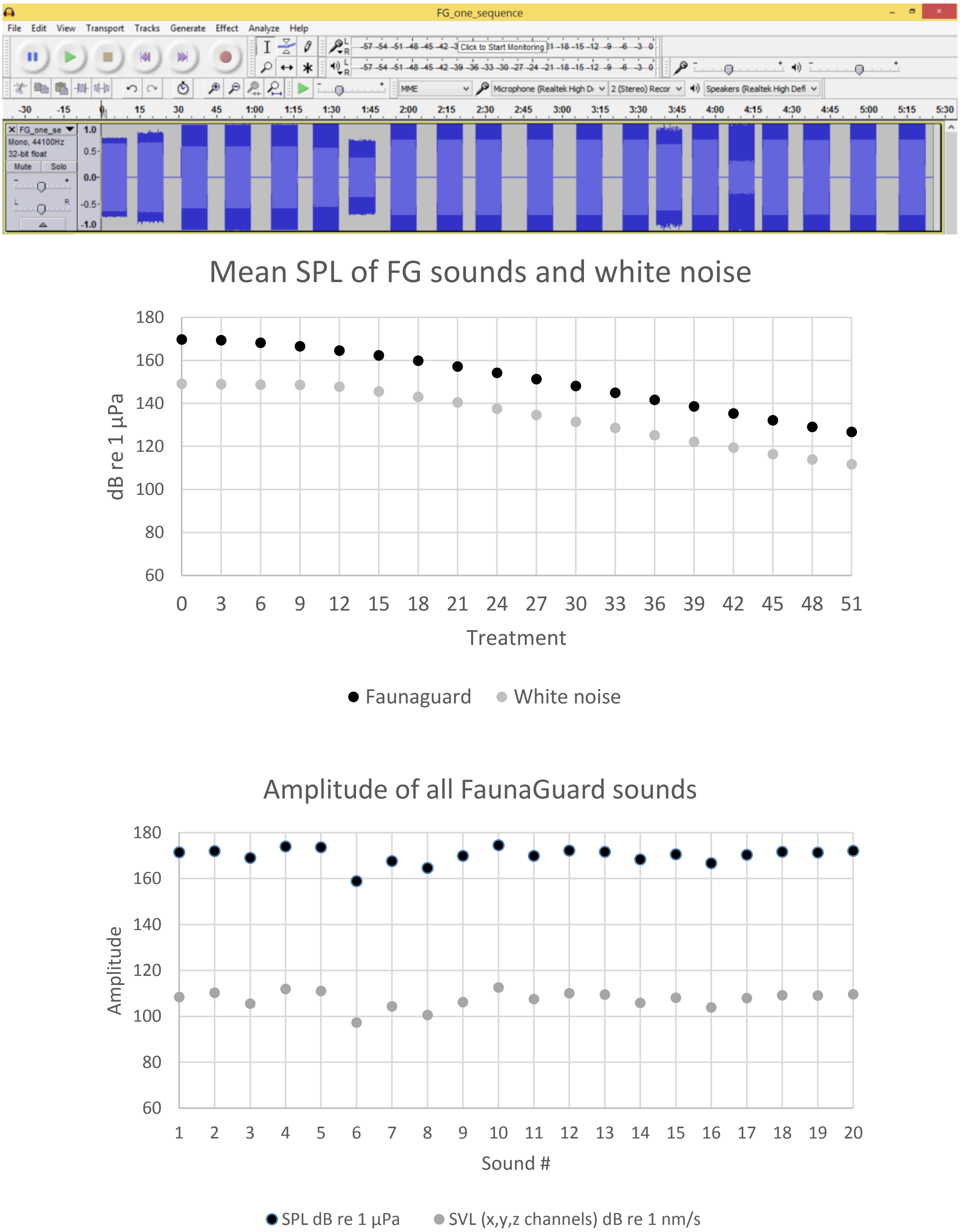


Figure 10: Amplitude wave (top) of the complete FaunaGuard series of 20 sound stimuli (FM-FM displayed in Audacity); Mean sound pressure level (SPL) of all 20 sounds of the FaunaGuard (black) and white noise (grey) exposures (middle). The measured SPL are the mean of 12 recordings, two recordings for each of the six distances from the speaker, across the net pen; Amplitude (SPL and SVL) of all FaunaGuard sounds (again means of 12 recordings of the treatment ‘FG-0’, two recordings for six distances from the speaker, across the net pen).

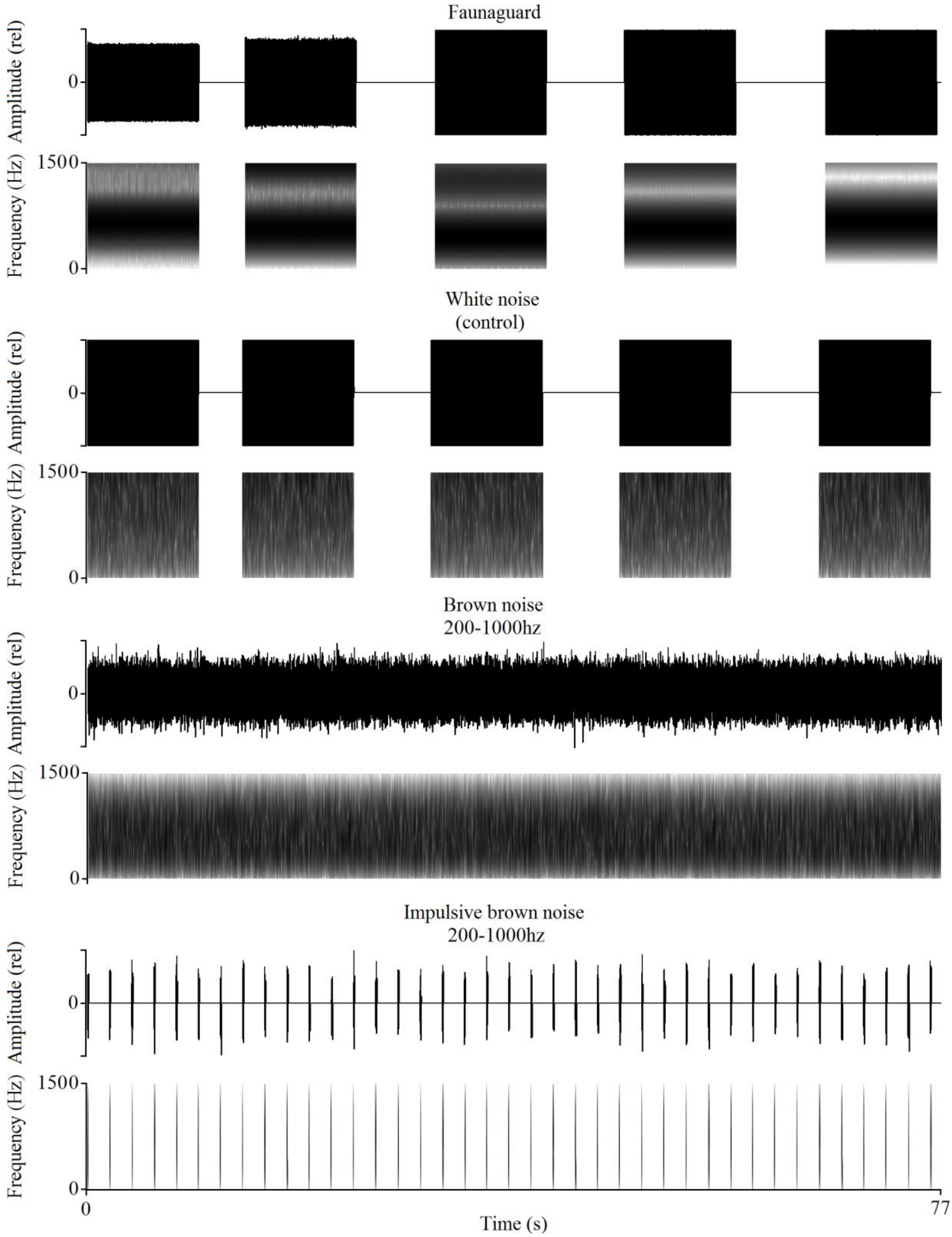


Figure 11: Amplitude wave forms and sonograms (generated in PRAAT) for the first five 10-second stimuli of the FaunaGuard (top) and the white noise control with the very same temporal pattern (3- to 10-second intervals) as used for the current experiments (directly below Faunaguard). ‘Brown noise’ and ‘Impulsive brown noise’ (bottom two) are depicted at the same temporal scale for comparison with the temporal patterns (continuous and 0.1-second pulse with 1.9-second interval) of these stimuli as used in previous experiments (Neo et al. 2016).

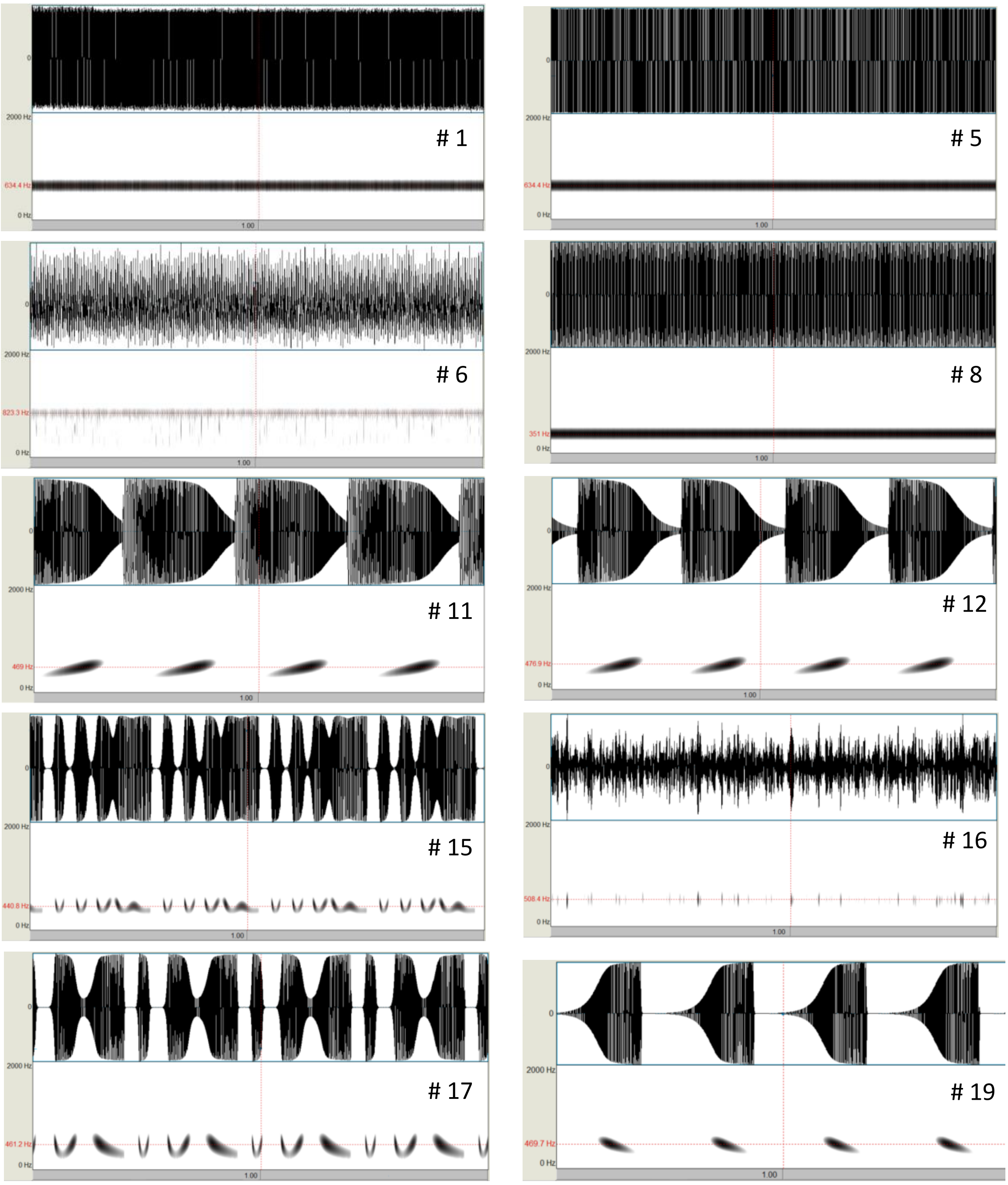
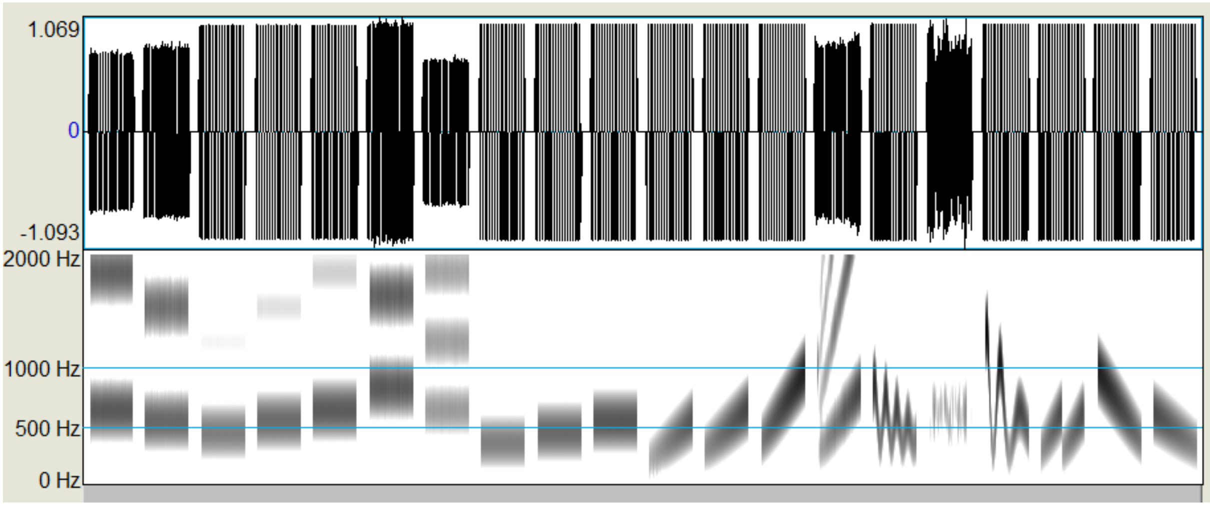


Figure 12: Amplitude wave forms and sonograms (generated in PRAAT) for a selection of ten FaunaGuard sound stimuli at a consistent temporal scale of 2 seconds and a spectral scale of 2.0 kHz. The upper four stimuli represent tones of different frequency (FG-FM #1 to #10). The examples of #11, #12, and #19 reflect variation in upsweeps and downsweeps; #15 and #17 are the two more variable frequency-modulated stimuli; and #16 is the noise band. The detailed description of all stimuli in words can be found in Table 1 and the acoustic contrast of subsequent sounds can be explored in Fig. 13).

Each group of fish was tagged at least two days (>40 h) before transfer to the net pen (Fig. 14). Transfer took place in a plastic container (60L) and the fish could acclimatize overnight, for at least 8 h. A group was exposed to three treatments per day, for two days. We conducted one trial at flood tide (starting 2:45 h before absolute high tide), one at high tide (starting 0:20 h before absolute high tide) and one at ebb tide (ending 2:45 h after absolute high tide). This schedule was chosen to ensure that the water level in the Jacobahaven was deep enough (>3 m) during the trials to maintain a fixed difference in depth between the tag receivers hydrophones).

The researchers arrived at the platform about 40 minutes before the start of the trial. Upon arrival, all equipment was switched on. Every trial took 40 minutes and consisted of a 30 minute sound exposure and 5 minutes of silence before and after the sound. After a group of fish was exposed to six treatments in two days, it was caught and sacrificed.



Frequency (Hz) Time (10 sec)

Figure 13: Amplitude wave form and sonogram of a cut of each of the 20 FG-FM sounds in the order of application. The blue line at 500Hz indicates the upper boundary of the spectral range of best hearing for European seabass and the blue line at 1000Hz indicates the upper boundary of hearing for this species. Note that all harmonics (many are beyond the scale) are beyond the hearing range of the model species and most marine fish species (and there is therefore no reason to assume that they will affect responsiveness).

2.5 Statistics

The received tag signals were processed on a computer using MarkTags v6.1 & AcousticTag v6.0 (HTI, US). This led to the x,y,z coordinates of the 3D swimming patterns of all fish. These coordinates were used to calculate swimming depth, distance from the speaker, swimming speed and average inter-individual distance (group cohesion). To test for behavioural responses, we used 5-minute-bin-averages of these parameters from before the sound exposure ('before') to right after the start of the exposure ('during1'), which provides insight into an effect of the onset; and before the end of the exposure ('during2') and after the end of the exposure ('after'), which provides insight into an effect of the termination of sound exposure; comparing 'during 1' and 'during 2' allows for testing habituation in any response measure within the exposure period (cf. Neo et al., 2014). We used Repeated measures ANOVA's and consequently TukeyHSD posthoc tests to statistically test the bin-averages. To capture the transient speed change we used 1-min-bins for the parameter swimming speed, this is in accordance with previous studies (e.g. Neo et al., 2016).

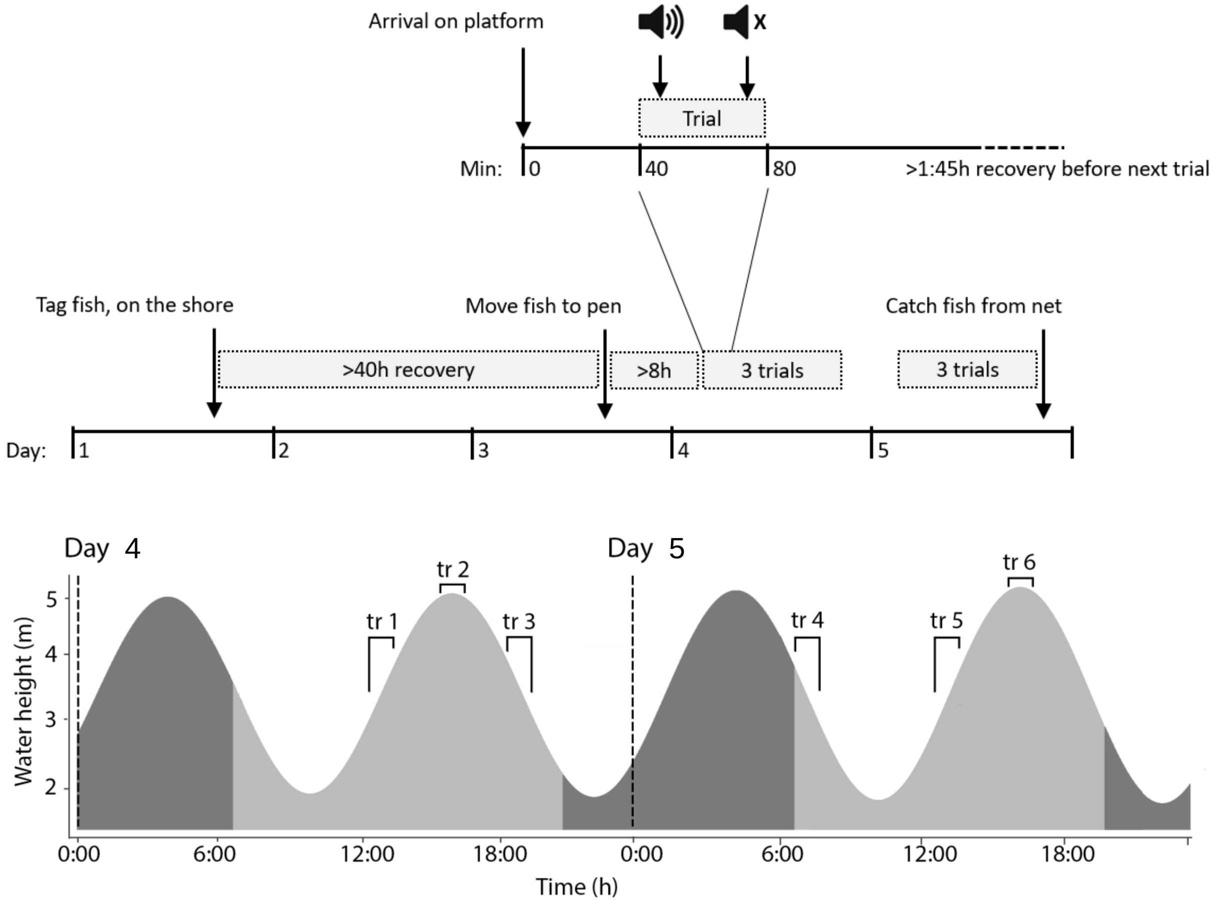


Figure 14: Timetable for the five-day experimental processing of a single group of four fish (top panel). Tagging was done on day 1, transition to the floating pen on day 3, and six half-hour sound exposure trials took place on day 4 and 5. We conducted three trials per day, before, during and after absolute high tide (bottom panel).

3. Results

3.1 Qualitative description

We have been able to describe detailed spatial patterns, at high resolution (Fig. 15), revealing distinct patterns of fluctuations in all four parameters (depth, distance from speaker, swimming speed and group cohesion, Fig. 16a and Fig. 16b). The sixteen groups of four fish typically swam up and down in the net pen from close to the surface to close to the bottom and were mostly between 1.0 to 4.0 m depth. Also in the horizontal pane, they explored the whole net pen, which is reflected in more or less cyclic patterns between 9.0 and 19.0 meters away (3D) from the speaker (Fig. 17). Cyclic patterns only occur when the four fish have formed a reasonably cohesive school and swim more or less in circles. Irregular patterns can reflect a school on a more random swimming path, or the mixture of individual paths, that can be regular circles but at different distances from the speaker (with some approaching and others receding) or with one or more individuals on a more random trajectory. Average swimming speed ranged from about 0.1 to 1.0 m/s, with fast groups (such as in G13 FG33) swimming mostly between 0.4 and 0.5 m/s and slow groups between 0.1 and 0.2 m/s (G17 FG30). Group cohesion fluctuated roughly between 0.5 and 8.0 m on average among pairs of the four group members.

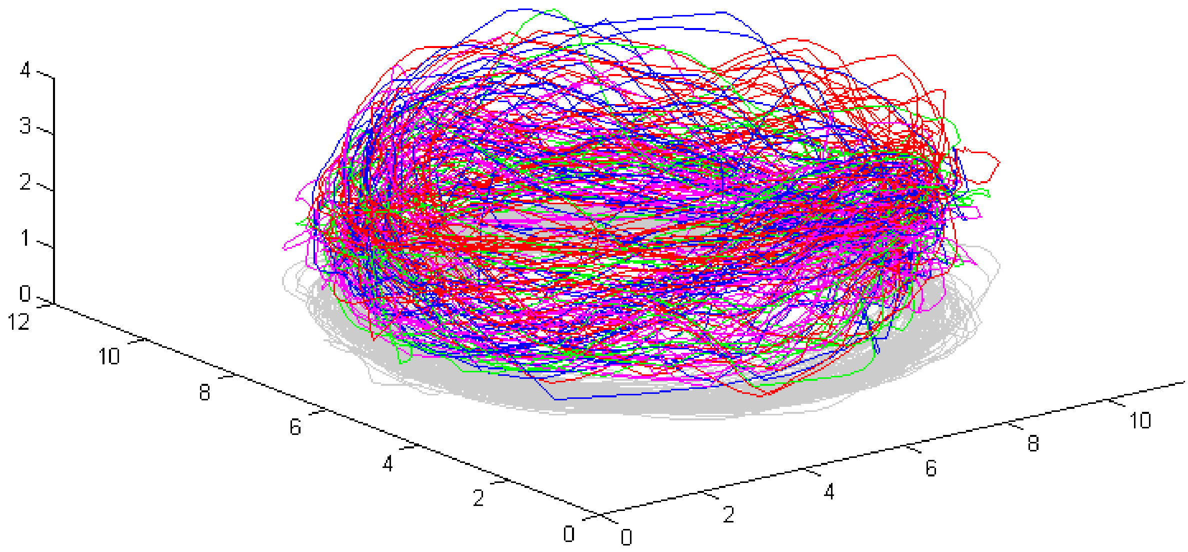


Figure 15: A 3D-reconstruction plot of the telemetric tracking with the HTI-system. Each colour is one of the four fish. The axes represent meters in the x, y, and z-direction and the shape of the accumulated trajectories reflects the cubic space exploited for swimming by the fish during a trial within the net pen. Swimming depth, 3D-distance from the speaker, swimming speed, and group coherence can be calculated for every instant in time during the 40 minute trial.

A 40 min trial consisted of a bit less than 6 times looped playback from minute 5 until 35 into the trial (Fig.14), which could either concern FaunaGuard or white noise sound sequences. We analysed behavioural patterns throughout this period and zoomed in to the periods before and just after on-set and off-set to investigate sound level dependent response tendency. Some distinct switches coincided with the onset of sound, which could indicate that the fish at least heard the sounds. Especially for the four highest sound levels of playback, there were such qualitative indications (Table 2): in 22 trials for 11 group (for each one trial with FaunaGuard and one with white noise sound series), we identified 7 downward and 4 upward shifts in the water column. In six out of the 22 trials, we scored a spatially deterrent effect and in two out of 22 a phonotactic pattern (approaching the sound source). In only two groups, we detected a brief spike in swimming speed that could reflect a distinct startle response for multiple if not all four of the fish (startle or acceleration, as startle in the narrow sense occurs within a second and will not be detectable at the current resolution). In six other groups, a pattern of minor acceleration could reflect startle responses for one or two of the fish or a general rise in swimming activity of all four. In three groups we even found a minor drop in swimming speed and in half of all 22, there was no indication of change. In nine out of 22 trials, fish groups had suddenly split up or at least one or more individual fish must have diverged in swimming paths, while five groups showed the opposite with a distinct clustering upon sound exposure.

3.2 Quantitative comparisons and statistical tests

Neither the FaunaGuard nor the white noise control sound series triggered consistent changes in behavioural patterns: as described above for the highest sound levels, always less than half of the groups showed any changes related to sound onset and these were often not congruent among parameters and varied in direction among groups. Although there are also some suggestive examples, such as a sudden splitting up right after onset of the sound for G1 FG18 and right after turning off the sound in G8 WN24, for the other lower sound levels there were even less distinctive patterns visible. Furthermore, there are also many fluctuations that seem fully independent of the exposure treatment. There was for example a distinct shift upward in the water column between 7 and 8 minutes into the trial (2 to 3 minutes after the sound onset) for G9 FG30. There was also a distinct switch from being steady at similar depth, to swimming up and down the water column in G6 WN42 and another distinct switch from irregular fluctuations in the 3D-distance from the speaker to regular cyclic patterns took place at 25 minutes into the trial in G6 WN24. An example of a sudden and strong increase in swimming speed was also found at 25 minutes into the trial in G1 WN00, but this moment in time was not apparent in other behavioural parameters. Fish from trial G8 FG39 swam consistently close together and suddenly changed their pattern around 13 minutes into the trail (8 minutes after the sound onset) and went from an average of around 1.0 m to 6.0 and 7.0 m distance among each other.

Table 2: Qualitative interpretation of behavioural patterns for 11 groups that received one of the highest exposure levels (gain range 00-09). The trial code indicates the group number (e.g. G1), whether it concerns FaunaGuard sound (FG) or white noise (WN), and the gain level. The characterization of patterns concerns a subjective description for a single event for a group of four potentially interactive fish of the time period of several minutes before and several minutes after the onset of the sound exposure (5 minutes into the trial). There were minor and moderate shifts in the water column (up or down in depth), minor movements towards or away from the speaker or a delay in approach in cyclic distance patterns (stay away – after which indicated the temporal range in minutes into the trial), minor accelerations, brief spikes in swimming speed (indicative of a distinct startle response), minor to strong drops in swimming speed, and finally there were weak to strong changes in group cohesion, both fish getting close to each other (clustering) and getting away from each other (fission).

Trial code	Depth	Distance	Speed	Cohesion
G1 FG00	None	Stay away 8-14	None	None
G1 WN00	None	Stay away 12-16	None	Weak fission
G3 FG03	Minor down	None	Minor acceleration	Weak fission
G3 WN03	Minor down	None	None	None
G6 FG06	Minor down	Minor approach	Minor acceleration	Strong clustering
G6 WN06	Minor down	Minor away	None	Moderate fission
G7 FG09	Moderate up	None	None	Weak fission
G7 WN09	None	Minor away	None	Weak fission
G8 FG00	None	None	Minor drop	Strong clustering
G8 WN00	Minor up	Stay away 6-7	Brief spike up	None
G10 FG03	None	Minor approach	Minor drop	Moderate fission
G10 WN03	None	Minor away	Minor acceleration	None
G12 FG06	Minor down	Minor stay close	Brief spike up	None
G12 WN06	None	None	None	Weak clustering
G13 FG09	None	Pattern disrupted	Strong drop	Strong fission
G13 WN09	None	None	Minor acceleration	Weak clustering
G14 FG00	None	None	None	None
G14 WN00	None	None	None	Weak fission
G16 FG06	Minor up	None	None	No data
G16 WN06	Minor down	None	Minor acceleration	No data
G17 FG09	Minor up	None	None	Weak fission
G17 WN09	Minor down	None	Minor acceleration	Strong clustering

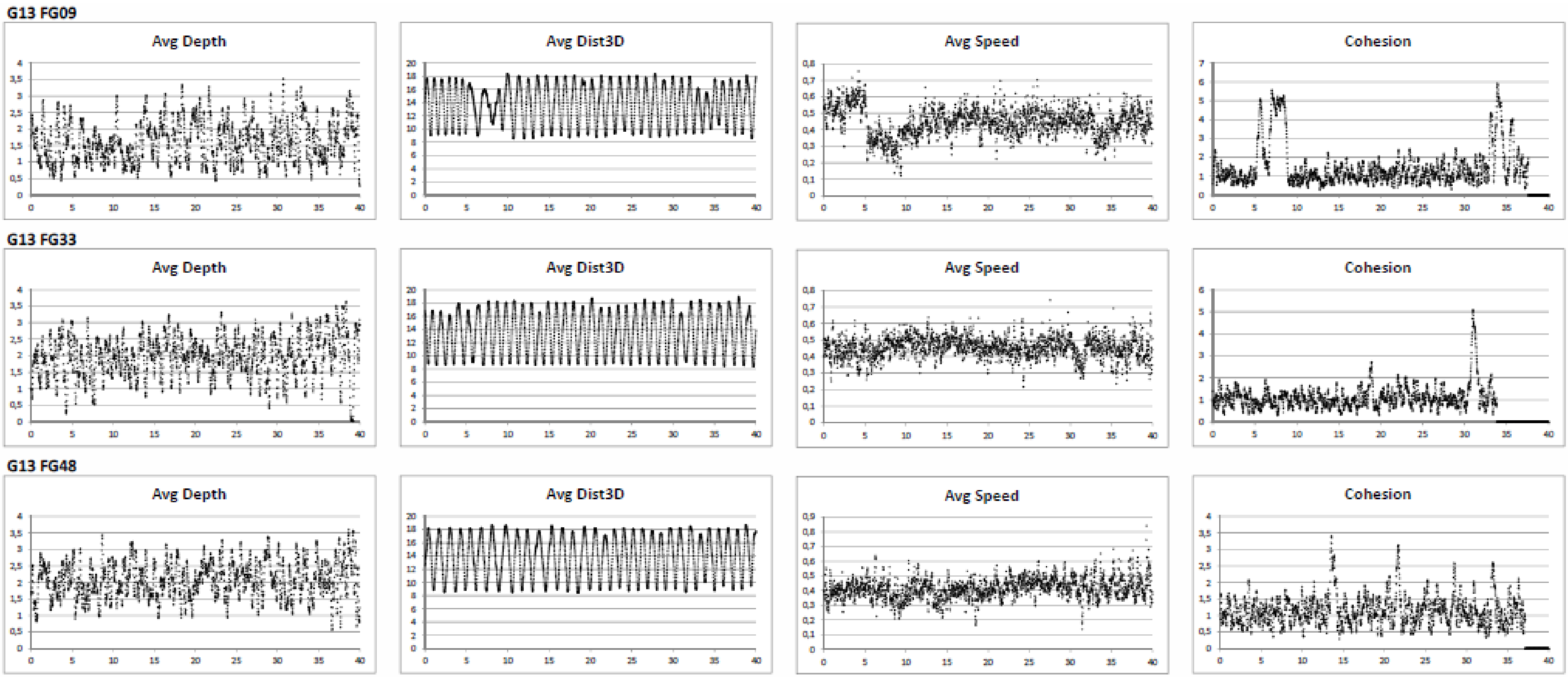


Figure 16a: Results of group 13 for the three sound exposures of the FaunaGuard sounds. All y-axes show results in meters or meter per second (speed). The x-axes show the time in minutes. The graph ‘Avg Depth’ shows the average distance from the bottom of the grid during a trial. ‘Avg Dist3D’ shows the average distance from the speaker (in 3D). ‘Avg Speed’ shows the average speed. ‘Cohesion’ shows the average inter-individual distance. The exposures took from minute 5 to 35 and were preceded and followed by 5 minutes of silence. The typical fluctuation pattern that can be seen in distance from the speaker represent the fish swimming in circles along the octagonal pen. A possible response behaviour after the start of the sound exposure can be seen in trial FG09: the average swimming speed decreased abruptly, the group cohesion suddenly increased (school fission) and the cyclic pattern of fluctuation in distance from the speaker was disturbed and became less extreme and slower.

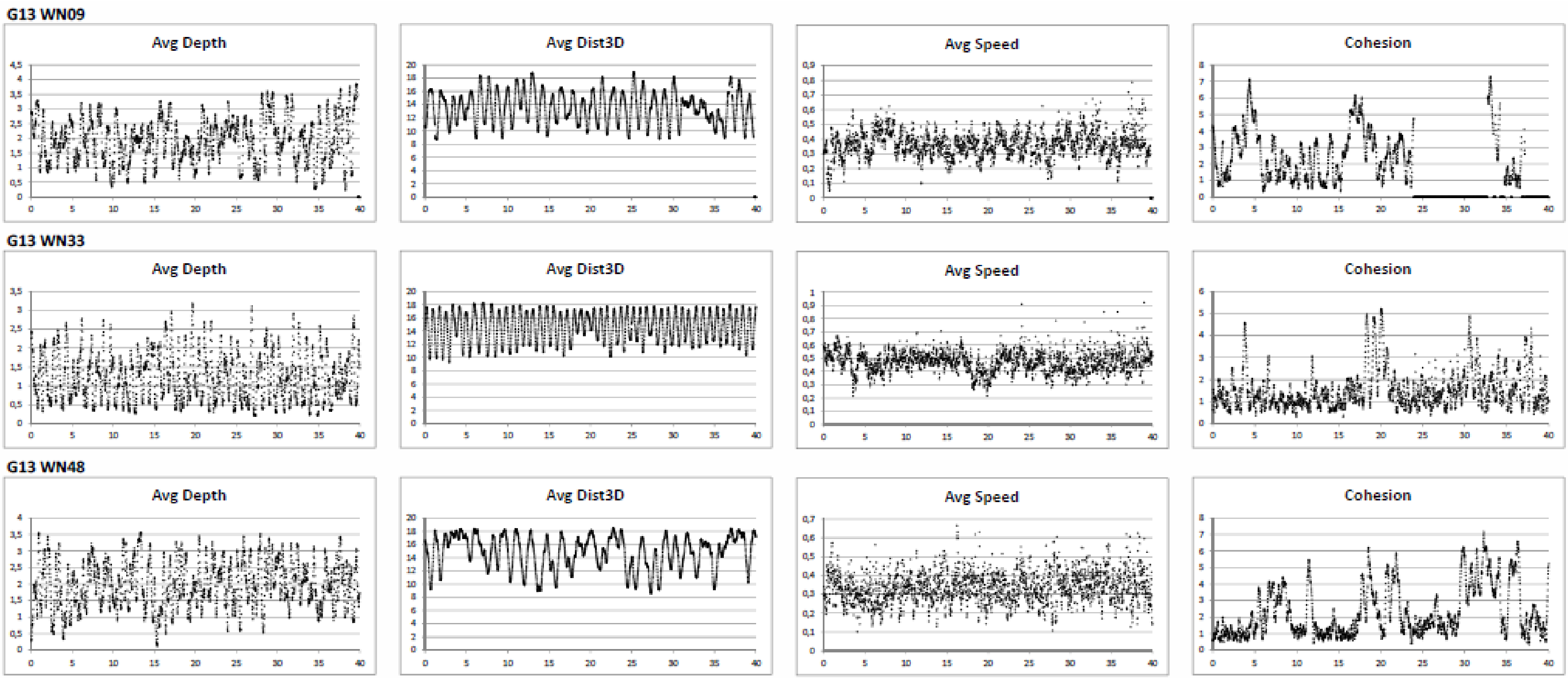
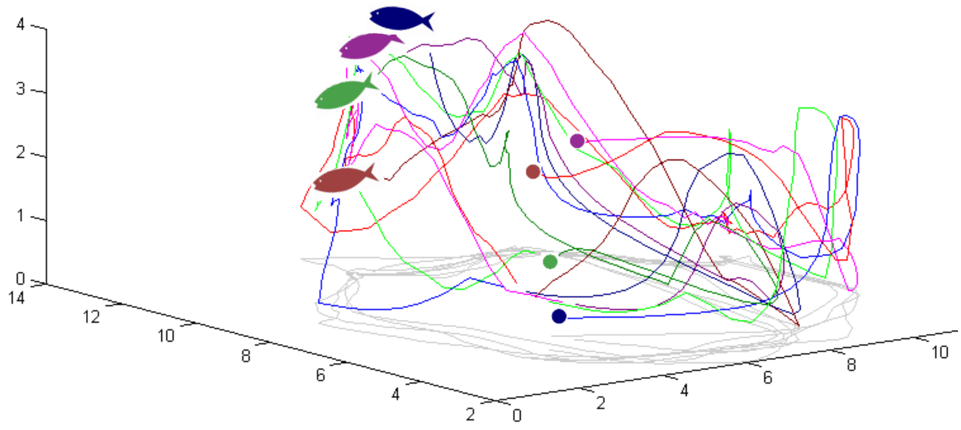


Figure 16b: Results of group 13 for the three sound exposures of the white noise control sounds. All y-axes show results in meters or meter per second (speed). The x-axes show the time in minutes. The graph ‘Avg Depth’ shows the average distance from the bottom of the grid during a trial. ‘Avg Dist3D’ shows the average distance from the speaker (in 3D). ‘Avg Speed’ shows the average speed. ‘Cohesion’ shows the average inter-individual distance. The exposures took from minute 5 to 35 and were preceded and followed by 5 minutes of silence. Possible changes in behaviour after the start of the sound exposure can be seen in group cohesion of the trials WN09 (clustering: decrease in cohesion, after a pre-exposure increase) and WN48 (fission: increase in cohesion, which means that one or more individual fish enlarge the distance to the others).



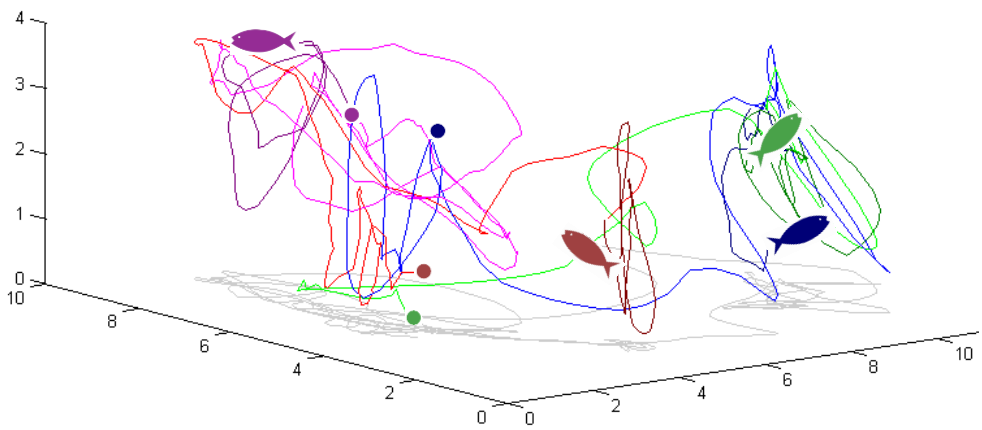


Figure 17: Two examples of typical swimming patterns in 3D-reconstruction plots of the telemetric tracking. Each colour is one of the four fish: the track line starts at the dot and changes from light to dark with time until the final position, indicated with a fish. The axes represent meters in the x, y, and z-direction and the grey track lines reflect the accumulated trajectories in 2D at the bottom. The group in the top panel (G1 FG00) swam in a school up and down, but in relatively consistent left-turning circles (check grey 'shadow' lines at the bottom). The group in the bottom panel (G6 FG06) concerns an example where the four fish swam relatively independently and repeatedly swam up and down somewhere at the net. All groups are included in the data processing and statistical analyses to test for consistency of any pattern.

For a more quantitative comparison and statistical testing we binned five minute periods for swimming depth, 3D-distance from the speaker and group cohesion and one minute periods for swimming speed. This allowed explicit exploration and tested for any change from before to during sound exposure, from the start to the end of sound exposure, and from the end of sound exposure to just after sound exposure ended. Any consistent patterns across the 16 groups across sound levels should stand out as they have in previous experiments for swimming depth in particular (Neo et al. 2016). On the contrary, we found no significant variation for any of the four parameters, neither for the FaunaGuard nor for the white noise sound series (ANOVA, all $P > 0.4$, most $P > 0.9$). This is true for all sound levels combined (Fig. 18a) and also if only trials with the highest sound levels were included (Fig. 18b).

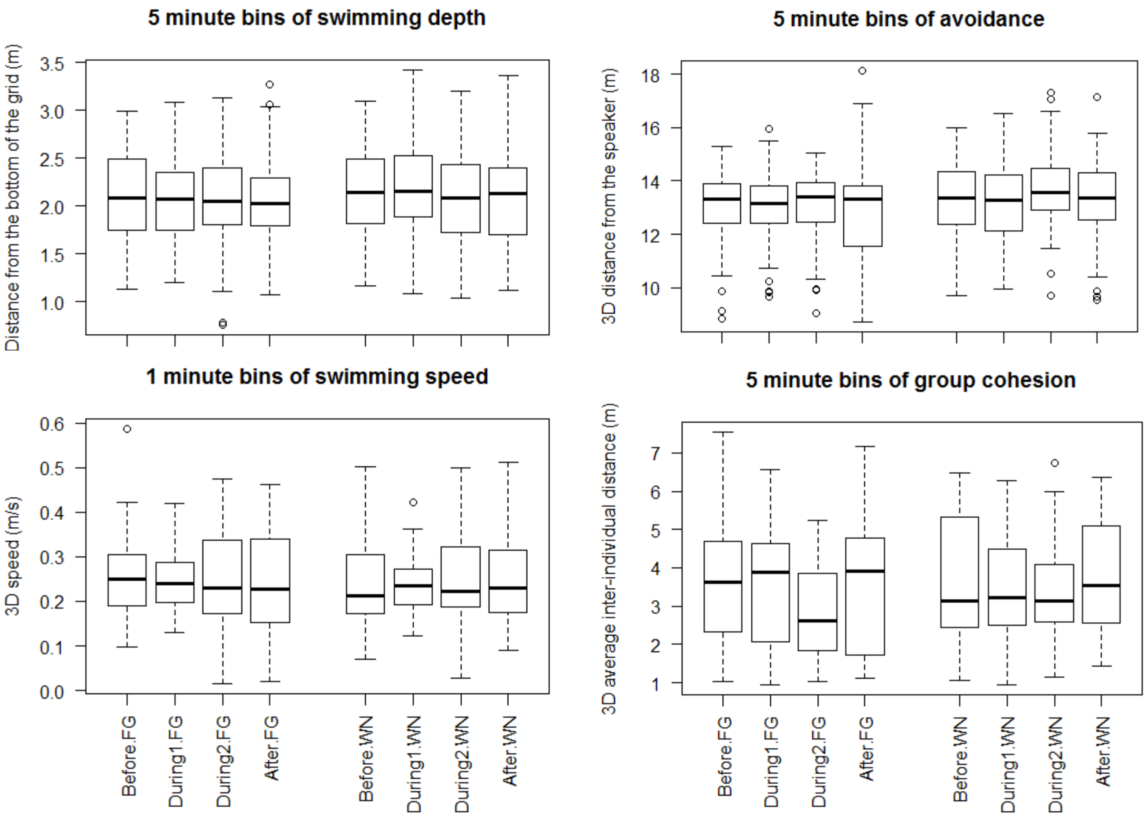


Figure 18a: Overall results, all sound levels accumulated, on behavioural responsiveness to sound exposure for 16 groups of four seabass. Box-whisker plots reflect the median, upper and lower quartiles, and extreme values. In each graph, the four time periods on the left depict measurements for the FaunaGuard (FG) sound series and the four time periods on the right depict measurements for the white noise control sound series (WN). The 'Before'-measurements concern the 5 or 1 minute bins just before sound onset, 'During1' concerns the 5 or 1 minute bins just after onset, 'During2' the 5 or 1 minute bins just before the sound is turned off and 'After' concerns the 5 or 1 minute bins without sound just after sound exposure has ended. Statistical tests indicated no significant variation among bins (N = 16 groups, 48 trials).

We also zoomed in on the potential startle responses to individual sound stimuli by binning very short 10 second periods, just before and during all the 20 sound stimuli in a series (Fig. 19). However, we did not find any distinct pattern for a particularly salient sound stimuli among the sounds of the FaunaGuard or the white noise control sounds. There were no patterns that could indicate a consistent immediate effect of a particular sound, nor any obvious patterns in the response to the sequence of sounds (order and interval variation was always the same). If we considered only the sound presentations of the loudest category, there were also no immediate stimulus or sequence effects. Finally, we also plotted the behavioural response patterns per sound level, accumulated for all FaunaGuard sounds and the white noise control sounds (Fig.20). However, also there we saw no consistent responses to any of the sound levels and consequently also no effect of sound level on responsiveness.

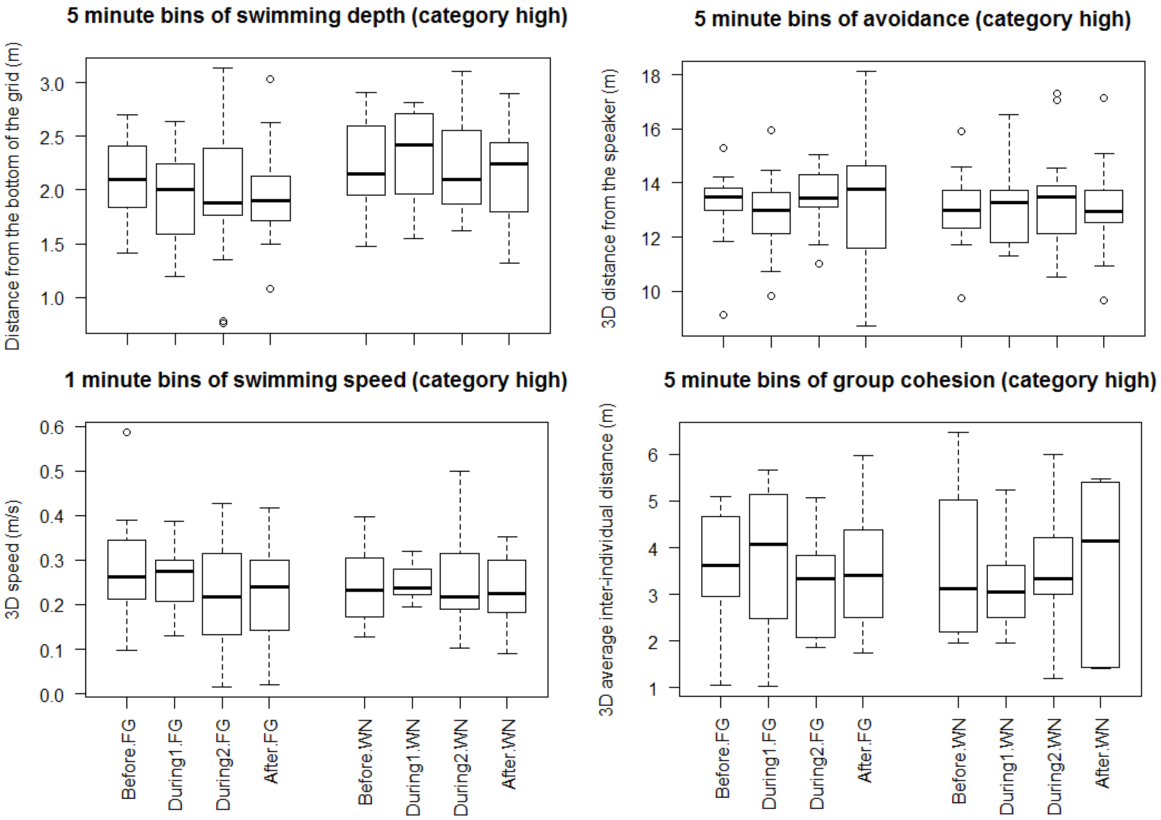


Figure 18b: Overall results, but only for the high sound level category trials, on behavioural responsiveness to sound exposure for 16 groups of four seabass. Box-whisker plots reflect the median, upper and lower quartiles, and extreme values. In each graph, the four time periods on the left depict measurements for the FaunaGuard (FG) sound series and the four time periods on the right depict measurements for the white noise control sound series (WN). The 'Before'-measurements concern the 5 or 1 minute bins just before sound onset, 'During1' concerns the 5 or 1 minute bins just after onset, 'During2' the 5 or 1 minute bins just before the sound is turned off and 'After' concerns the 5 or 1 minute bins without sound just after sound exposure has ended. Statistical tests indicated no significant variation among bins (N = 16 groups, 16 trials).

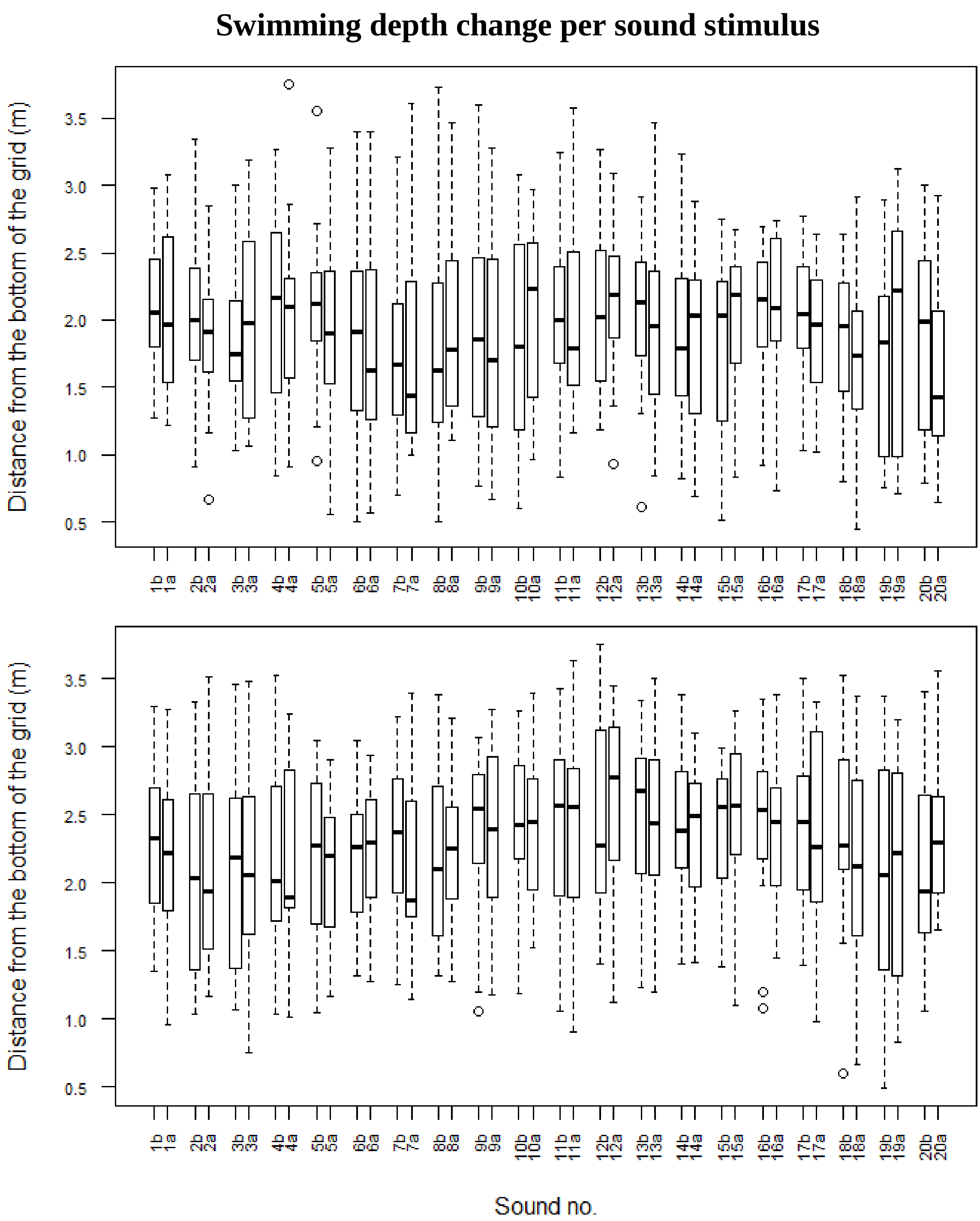


Figure 19: Response patterns throughout the sound sequence, all sound levels accumulated, for each of the 20 sound stimuli of the FaunaGuard (upper panel) and the white noise control with exactly the same temporal pattern (lower panel). A 'b' stands for before and an 'a' for after and refers to 10 second bins just before and during exposure to the respective sound stimuli (1-20, only first exposure series considered in trial with multiple series). A description of all FG sound stimuli can be found in Table 1 and sonograms of sounds are depicted in Fig. 11, 12, and 13. Each sound is the same for the white noise sound series. Statistical tests indicated no significant variation among 'b' and 'a' bins for any of the sound stimuli (N = 16 groups, 16 trials, for both treatments).

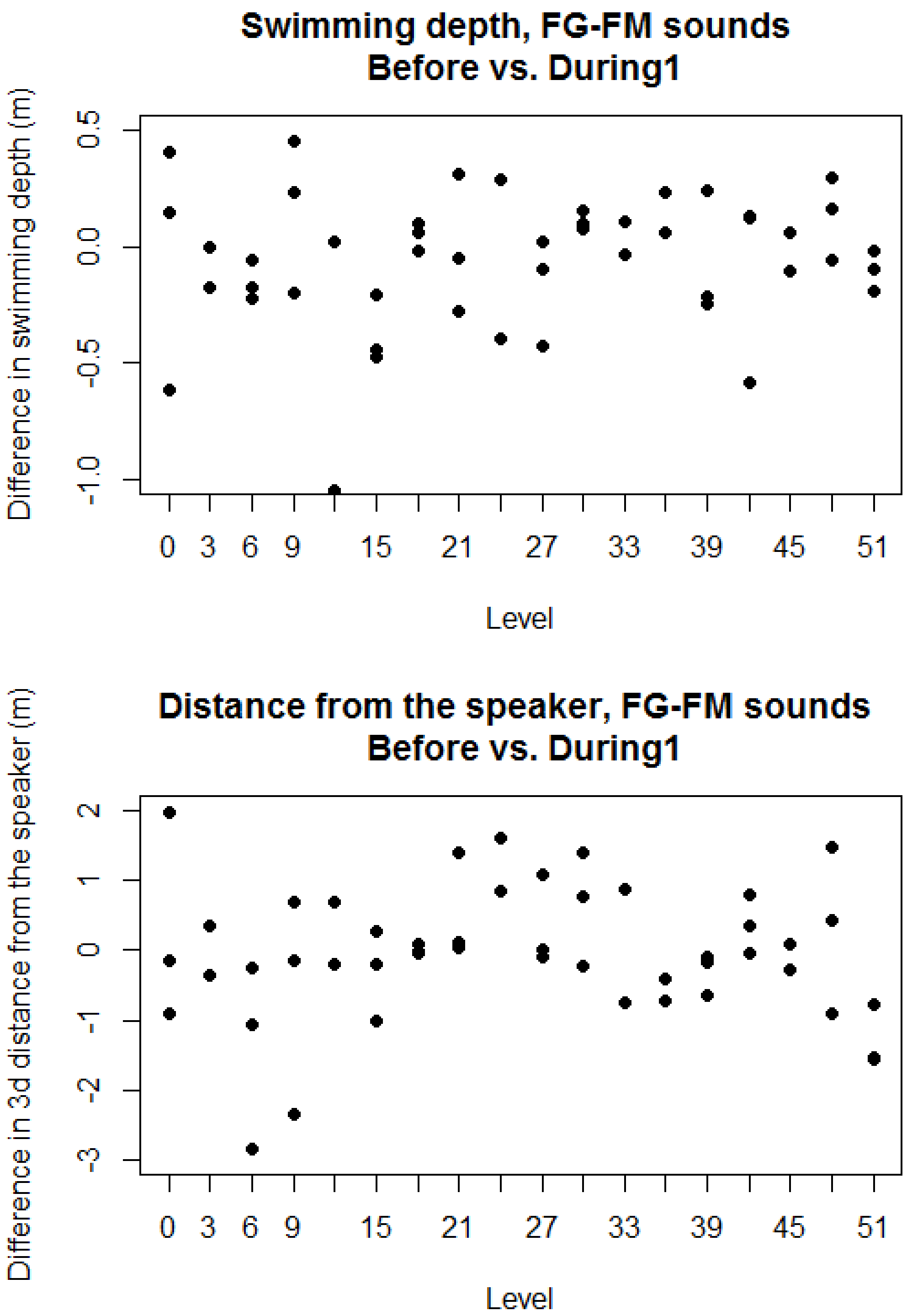


Figure 20: Response patterns for swimming depth (upper panel) and 3D distance to the speaker (lower panel), all sound stimuli of the FaunaGuard accumulated, for each of the 18 sound levels separately (N = 16 groups, n = 48 trials, 2 or 3 per sound level). The closed circles represent the difference in swimming depth or distance from the speaker between the first 5 minutes after the start and the last 5 minutes before the start of the treatment. There is no consistent sound level dependent pattern for increase (>0) or decrease (<0) in depth or distance, even though there are quite a few groups that approach the sound source at the highest sound levels (Sound pressure levels for each gain level are reported in Fig. 10).

4. Discussion

The results of the tests with the sounds of the FaunaGuard Fish Module (FG-FM) were unexpected. A range of sound levels, from very faint to at least equally loud to what has triggered significant behavioural changes in previous experiments (Neo et al. 2016; Neo 2016), did not elicit consistent changes in swimming patterns. Occasional switches in depth, distance, speed or group coherence suggested that the fish did hear the sound exposure, but in none of our general tests or more detailed data explorations we found any evidence for statistically significant behavioural effects. There was no effect of fish behaviour from the FaunaGuard as a whole, by specific sound stimuli in particular, or by the white noise control sounds. As even the loudest exposures did not induce a change in any of the parameters, we could not provide a dose-response analysis. We therefore conclude that the FG-FM sounds, at the maximum sound levels tested and for the current species and batch in a net pen, does not trigger a vertical downward shift in the water column nor a horizontal avoidance response. We also have no strong indication that the FG-FM at high, moderate or low sound levels would attract fish. The discussion will address potential reasons for the results and we suggest potential ways to improve the efficacy of the FG-FM as an Acoustic Deterrent Device (ADD).

4.1 Fish species and batch

There is no evidence that seabass would be affected in their behaviour, or would move away from the sound source, in the area surrounding the FaunaGuard in which the sounds are audible but not above the maximum currently tested. It could still be that more close to the FaunaGuard, or at sound levels above the maximum currently tested, the FG-FM sound series may have a spatially deterrent effect on seabass or make them move down the water column. Furthermore, given the anecdotal evidence from efficacy of the FG-FM during field applications, it could be possible that 1) free-ranging fish respond differently and stronger than captive fish in a net pen (Neo et al. 2016; Bruintjes et al. 2016); and that 2) fishes from other species or background than hatchery-reared seabass respond differently and stronger (Neo et al. 2015). It has for example been reported that different cohorts of hatchery rainbow trout (*Oncorhynchus mykiss)* can have different hearing threshold (Wysocki et al. 2007), which obviously could also affect responsiveness, on top of potential differences in general behavioural tendencies (Shafiei Sabet et al. 2015). Future testing in free-ranging fish or with fishes of different species and background in a net pen is needed to exclude or confirm these uncertainties.

4.2 Amplitude of exposure

An important factor to consider is the amplitude of exposure. There will always be a threshold sound level above which there will be a behavioural response, but that level has not been reached in the current experiment. All FaunaGuard treatments in the loudest category were played back at sound pressure levels above 160 dB re 1 µPa. This is slightly lower than the 165 dB re 1 µPa for sounds used in a previous experiment in the same net pen and the same species of fish (Neo et al. 2016). However, many of the FaunaGuard sounds in the three loudest gain levels were above 170 dB re 1 µPa, which is louder than the treatments in the previous experiment (Neo et al. 2016). The startle response tests in the fish tank at SEAMARCO were done for a few sounds at about 150 dB re 1 µPa, but these only triggered a response in 25-50 % of the tests and were adjusted (in frequency) or replaced for the FG-FM upgrade that we currently tested. Other sounds were all played in the range of 170-190 dB re 1 µPa and were successful in triggering high response tendencies, except for when it concerned frequencies of 1000Hz or higher (Kastelein et al. 2011).

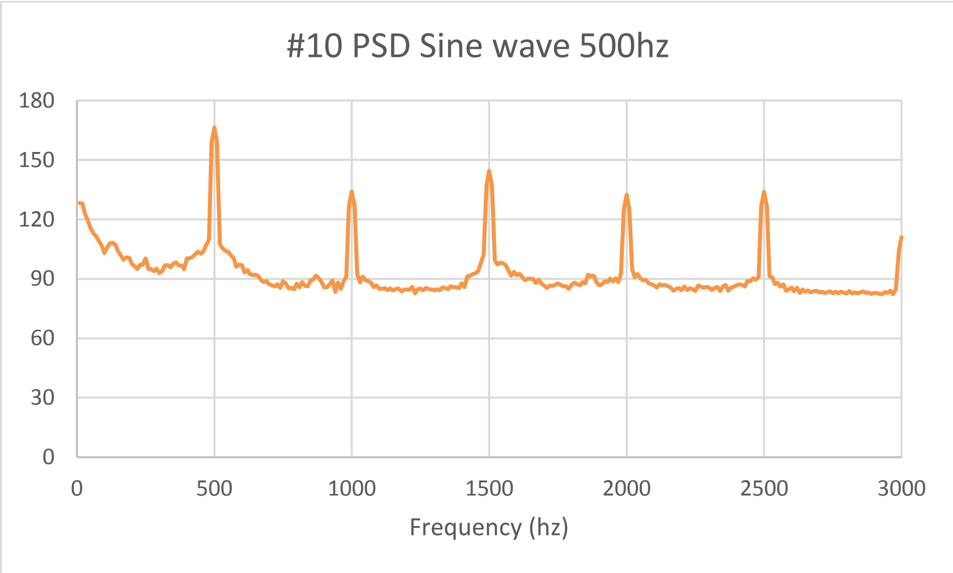


Figure 21: Power spectral density (PSD) graph of FG sound stimuli # 10 . The units on the y-axis refer to sound pressure level in dB re 1 µPa/Hz. The harmonic structure of the sine wave of #10 clearly shows as amplitude peaks at multiple integers of the fundamental frequency at 500Hz. Seabass are most sensitive in the range of 100-400 Hz and also hear up to 1000 Hz (see Fig. 3). PSDs for all other FG-FM stimuli are provided in the appendix.

In the fish tank, the higher exposure levels triggered a startle or brief acceleration in a high percentage of the cases, but we cannot state that the five groups of two fish species responded more than in the current experiment. In the net pen, a startle may also have taken place, as these will occur within a time scale of seconds and may go unnoticed at the outdoor telemetric resolution. Furthermore, indoor and outdoor responses to sound exposure do not necessarily have to be different as has become clear from direct comparisons (Neo et al. 2016; Bruintjes et al. 2016). It is also important to keep in mind that startle responses are not necessarily related to more prominent behavioural changes or spatial shifts (down or away): examples of a lack of such a link can be found in open water (Wardle et al. 2001) and in fish tanks (Shafiei Sabet et al. 2015).

There are two factors that should be considered when evaluating sound level: the spectral range of hearing sensitivity of the seabass and the distance from the sound source. The current sound level measurements were processed for the frequency range audible to seabass 200-1000Hz and direct comparisons with Kastelein et al. (2011) depend on whether they did the same. If a wider range is used for such calculations, acoustic energy of harmonics that are not audible to the seabass will be included (see power spectral density graph in Fig. 21), which is better avoided. We used a laptop computer to send sounds to the FG-FM speaker in the current set-up and used the highest output levels possible without distortion becoming a factor. The FG-FM transducer (Lubell LL 1424) hung in the water at about 12 meters from the fish in the net pen (based on the middle of the net pen). Kastelein et al. (2011) also played the FG-FM sounds by a laptop computer to the same transducer, which hung in the water at about 4 meters from the fish in the fish tank. The FG-FM will be and has been used in outdoor conditions with a dedicated sound generator, which may be able to reach higher sound levels without distortion, but the target fish will also be further away from the source than 4 or 12 meters.

Sound levels will attenuate with distance depending on frequency and local propagation properties (e.g. Shapiro et al. 2009). A point source with equal sound propagation in all directions will attenuate at a rate of 6 dB per doubling of distance determined by the inverse square rule. Reverberations from surface and bottom will counteract this to a variable extent, while turbulence, large objects of different impedance or air bubbles will accelerate attenuation. The actual range of sound levels that free-ranging fish at various distances would be exposed to is best based on the source level of the FG-FM as used in practice and by application of sound propagation models that can take local conditions into account. We have very little to know insight into how free-ranging fish would respond to ADD sound exposure, but theoretically fish may move away, stay put, or move closer, and this is likely related to sound level of exposure, which fades with distance (see Fig. 5). We have tried to put the current results and those of Neo et al. 2016 in the framework of potential zones of effect to provide understanding in a potential explanation and to provide targets for follow-up studies (Fig. 22).

Ambient noise levels may also play a role in predicting behavioural responsiveness, in addition to the amplitude levels of the sound stimuli. Ambient noise levels in the SEAMARCO test basin were relatively low with a relatively flat level around 60 dB re 1 $\mu Pa^2/Hz$ between 150-400Hz (Kastelein et al. 2011), while these were considerably higher in the Jacobahaven and diminished from 90 dB re 1 $\mu Pa^2/Hz$ at 150Hz to 70 dB re 1 $\mu Pa^2/Hz$ at 400Hz (Figure 23). These levels are relatively high below 200Hz and comparable to recordings from fast-flowing rivers (Wysocki et al. 2007; and these low-frequency sounds may be generated here by water flow in the nearby Oosterschelde river), but still not very high for outdoor conditions above 200Hz (comparable or below levels in the fish tank of Vetter et al. 2015). Although we do not believe that noise levels can explain the discrepancy between the current results and those reported in Neo et al. (2016), as the location and season were the very same in these studies, open water conditions can be more noisy naturally and thereby potentially undermine a behavioural effect of hearing the FG-FM sound stimuli.

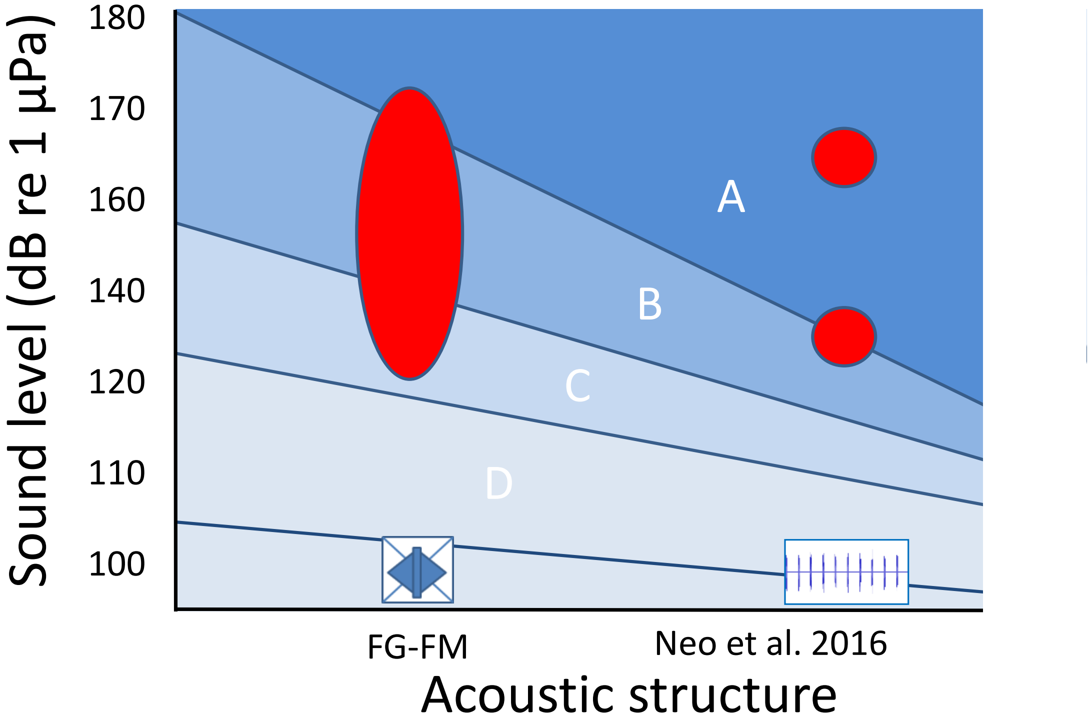


Fig. 22: Schematic illustration of a potential explanation for the discrepancy between the current lack of significant behavioural effects and the significant changes in behaviour to sounds exposure at relatively high and relatively low amplitude (Neo et al. 2016). The axes represent variation in acoustic structure on the x-axis and sound level of exposure on the y-axis. The blue zones correspond with the effect zones explained in Fig. 5. Zone A is where there is a significant target response; Zone B is where fish groups may be affected but do not move away or towards the ADD; Zone C is where fish exhibit phonotaxis; and Zone D is where the sounds are still audible, but do not elicit any spatial response. The two small red circles represent the two sound levels (low, ramp up, not exceeding 125 dB re 1 µPa in the first 5 minutes and high amplitude exposure at 165 dB re 1 µPa) of Neo et al. (2016). The large red oval represents the current test of FG-FM sounds which were tested at a wide range of sound levels, below, in between, and above those (from below 120 dB re 1 µPa to above 170 dB re 1 µPa , see Fig. 10). The behavioural response tendency is determined by a combination of acoustic structure and sound level; moving the FG-FM up in sound level or towards the pulse trains of Neo et al. (2016) may improve performance in the hypothetical framework that needs to be verified by future testing.

4.3 Spectral and temporal features

Given that previous experiments with different sound stimuli have triggered consistent response patterns at high, but also at very low levels, we argue that the FG-FM sounds (and the white noise control sounds) were not loud enough to elicit significant changes in behaviour, but that there are a number of acoustic features that may improve the efficacy of the FG-FM. Neo et al. (2016) tested the same species in the very same set-up and did not only elicit consistent and significant responses to a regular onset of a pulsed sound train, but also to a ramp-up procedure in which the sound levels did not rise over 125 dB re 1 μPa (Fig. 24). The reason for this apparently stronger triggering potential may be in the spectral composition (as they use brown noise instead of white noise, with more energy bias towards lower frequencies), the lack of tones as these have been reported to be less efficient in deterring fish than broad-band signals (Vetter et al. 2015, Fig. 23), or the shorter pulse duration and higher playback rate (shorter intervals), for which there are reports on efficient applications in fish deterrent devices (e.g. Dunning & Ross 2010; Gurshin et al. 2014).

5. Conclusion and recommendations

Given that previous experiments with different sound stimuli have triggered consistent response patterns at high, but also at very low levels, we argue that the FG-FM sounds (and the white noise control sounds) were not loud enough to elicit significant changes in behaviour in the current test conditions and set-up. However, the current findings and review do suggest that it is useful to further explore acoustic response tendencies of fish and that it is likely still possible to improve the deterrence capacity of the FG-FM sound stimuli, given that: a) previous experiments in the very same settings did trigger consistent responses at much lower sound levels (for seabass swimming down, not swimming away), b) that tones have been reported in the literature to be suitable for conditioning, but to be much less efficient in eliciting a spatial avoidance response than a complex, broad-band sound, c) that successful applications of acoustic fish guiding, as reported in the literature, often use brief and broadband sounds with relatively short intervals, and d) that we know still very little about the potential effect of using multiple sounds in a sequence.

We therefore recommend the exploration of effectiveness in triggering acoustic responsiveness to more brief (duration e.g. 0.1-0.5 sec) and broad-band (frequency range e.g. 200-1000Hz) sounds, repeated at relatively high rates (interval e.g. 0.1-2.0 sec). The potential to counteract habituation, of fishes that may be exposed for a longer period or repeatedly, may be explored by testing the effect of irregular variation in short and rapid and longer and slower pulse series within the ranges mentioned above. Also worthwhile to explore may be to occasionally insert longer intervals at irregular moments and of variable and unpredictable duration (interval e.g. 2.0-10.0 sec). Another factor to explore in this context is the use of multiple sounds, which could include brief broadband sounds of just the lower or upper part of the above mentioned range (e.g. 200-500Hz and 500-1000Hz), or the use of frequency-modulated sweeps or chirps across these ranges.

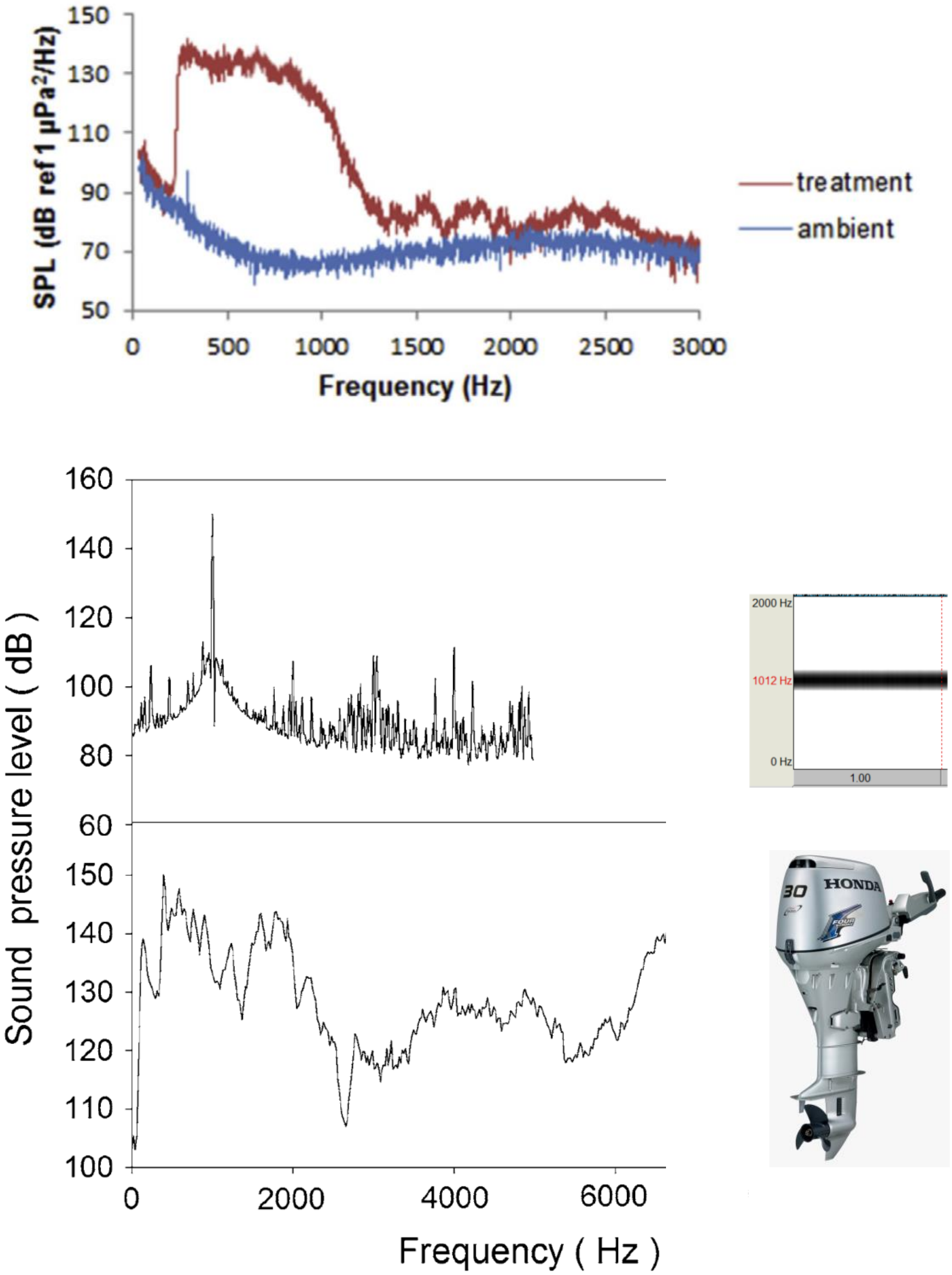


Figure 23: Power spectral density (PSD) graphs of the band-passed brown noise sound treatment and the ambient conditions at the Jacobahaven (upper panel, from Neo et al. 2016) and PSD graphs for a 1000 Hz tone and a complex broad band boat noise recording lower panel, from Vetter et al. 2015). The units on the y-axes refer to sound pressure level in dB re 1 μPa/Hz (see Fig. 2 for behavioural response data for these sounds).

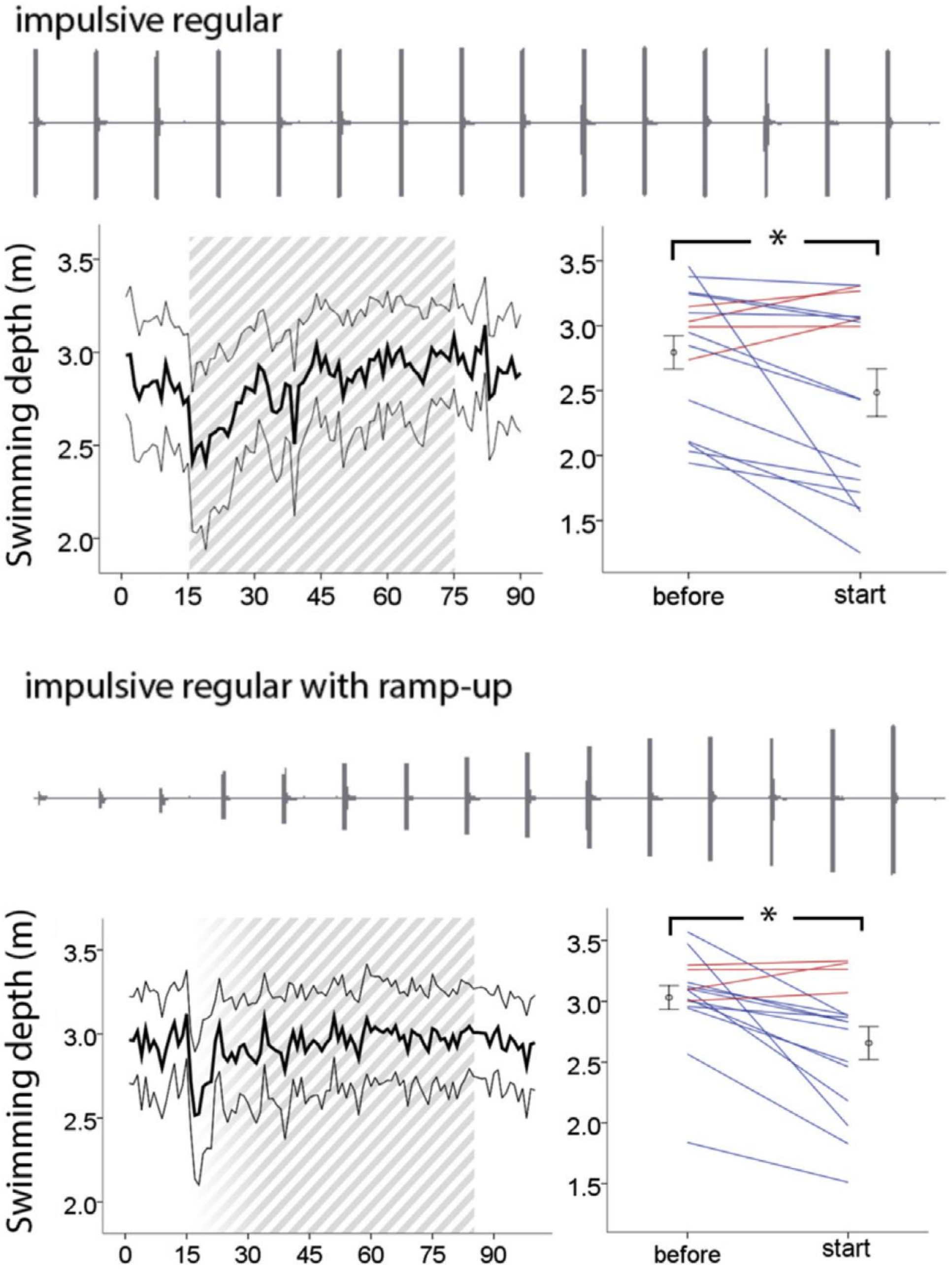


Figure 24: Behavioural response patterns for 16 groups of four seabass tested in the very same set-up as the current experiments (from Neo et al. 2016). Both an abrupt onset (upper panel) and a ‘ramp-up’ of 20 min (lower panel) for an impulsive signal (0.1 sec pulse duration, 2.0 sec duty cycle) yielded consistent behavioural changes. The time series plots for swimming depth indicate instantaneous mean levels in the bold lines (with 95% confidence intervals above and below). The shaded areas indicate the 60 minute noise exposure period. The paired comparisons on the right depict 5 minute bins from just before and just after onset of the sound exposure. Red lines depict fish that went up, blues lines fish that went down (mean and standard error also indicated). The fish in the abrupt onset treatment were exposed to pulses at a mean level of 165 dB re 1 μPa, while the fish in the ramp-up treatment did not reach 125 dB re 1 μPa yet in the first 5 min. An asterisk (*) denotes statistical significance ($F_{3,48}$ = 5.14 and 5.70, both $P < 0.005$, after Bonferroni correction for post-hoc comparisons).

In summary, we believe the most logical next steps for concrete tests would be:

1. Test same seabass batch to sounds of different temporal and spectral pattern in a net pen (like Neo et al. 2016) – done, data will be processed for Research report 2.
2. Repeat test of FaunaGuard sounds with other batch of seabass and add a more pelagic fish species – potential experiment for the net pen in the future.
3. Compare responsiveness to FG-FM sounds directly to pulse train like in Neo et al. (2016) at different sound levels to assess dose-response curve – can be combined with 2.
4. Investigate the effect of alternating or varying sounds in a sequence on response tendency and habituation – long-term plans, adequate acoustic contrast tests should be included.
5. Apply the FG-FM sound exposure at two distinct field sites with virtual source location of anthropogenic acoustic danger and monitor free-ranging fish by telemetry – critical for final evaluation of efficacy.
6. Assess source level of FaunaGuard and model spatial soundscape gradient for areas of application – not an explicit target of the current project and objectives and TNO-expertise.

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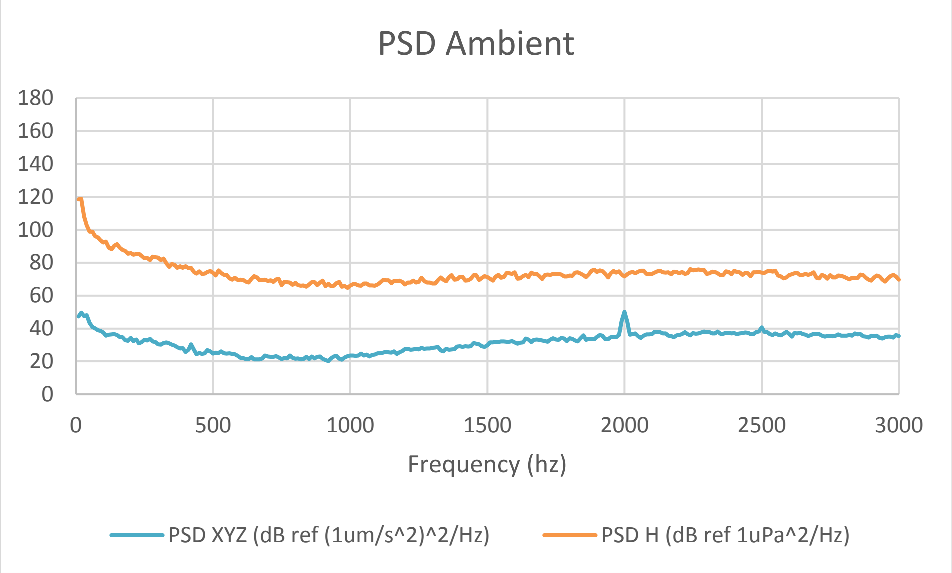
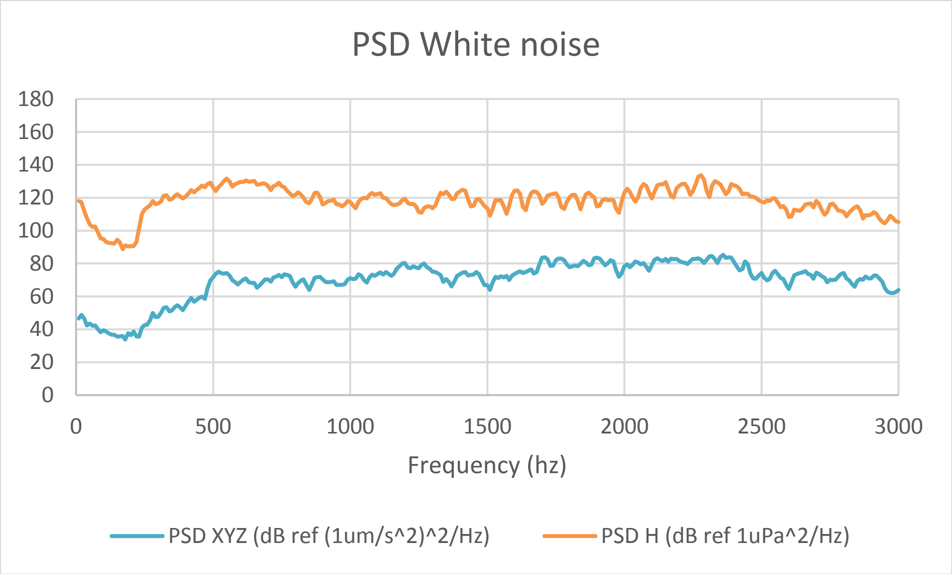
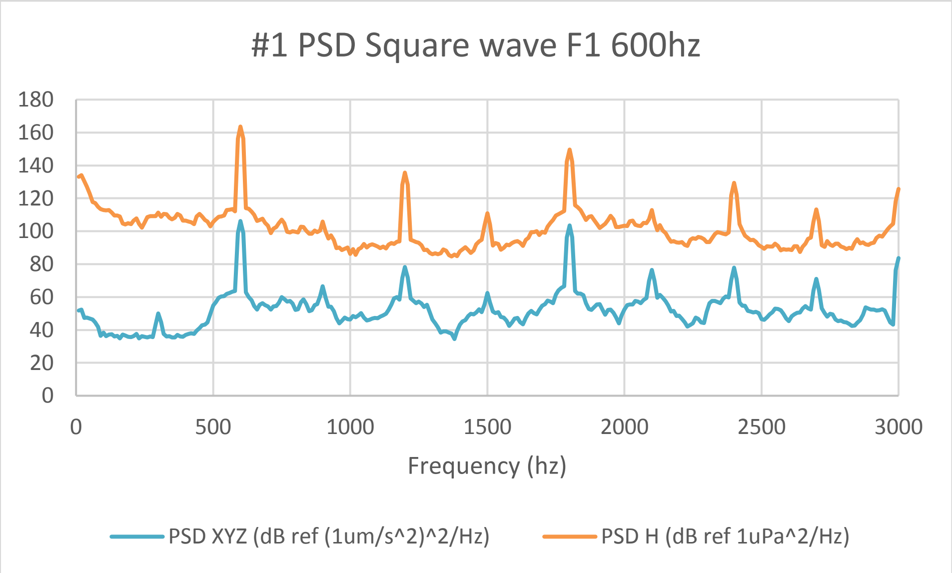
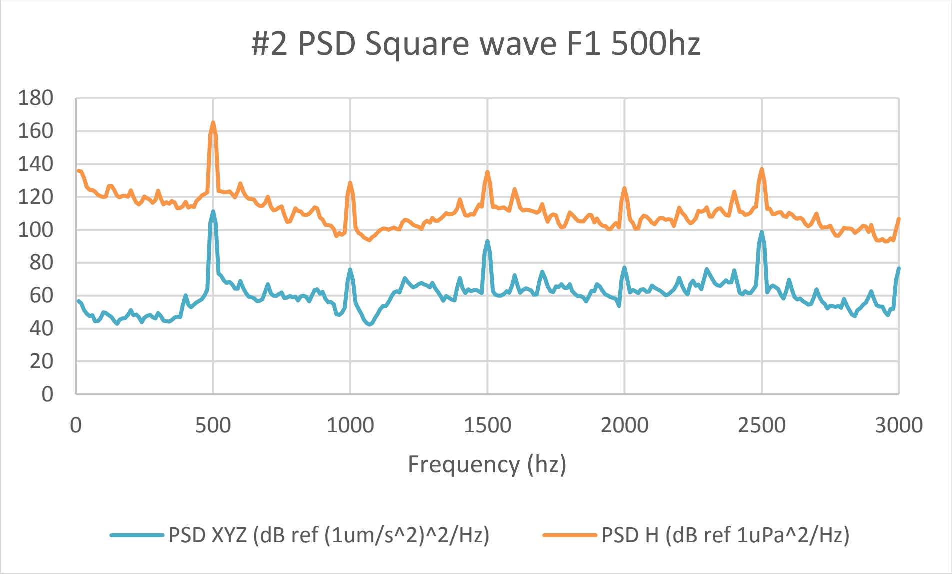
Appendix I: Power spectral densities





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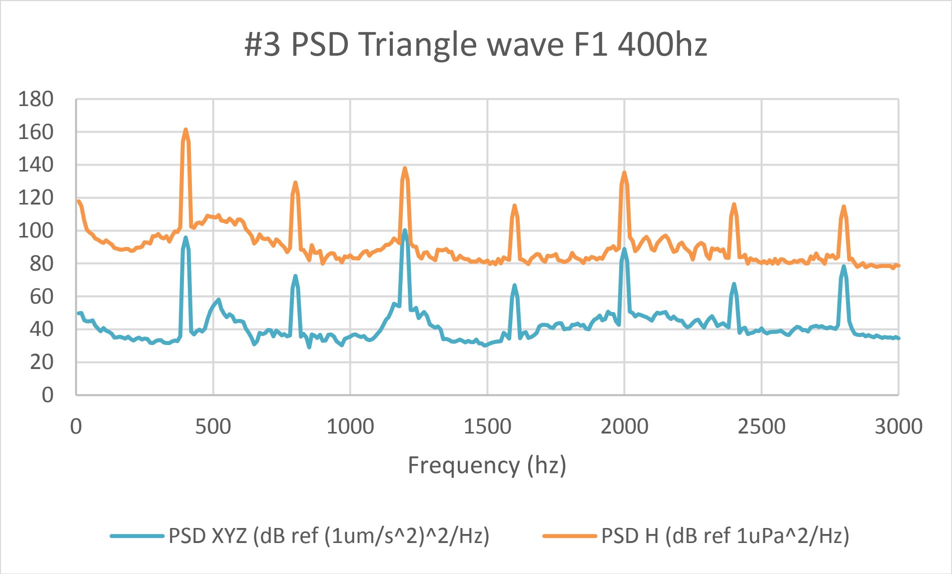
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#3 PSD Triangle wave F1 400hz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
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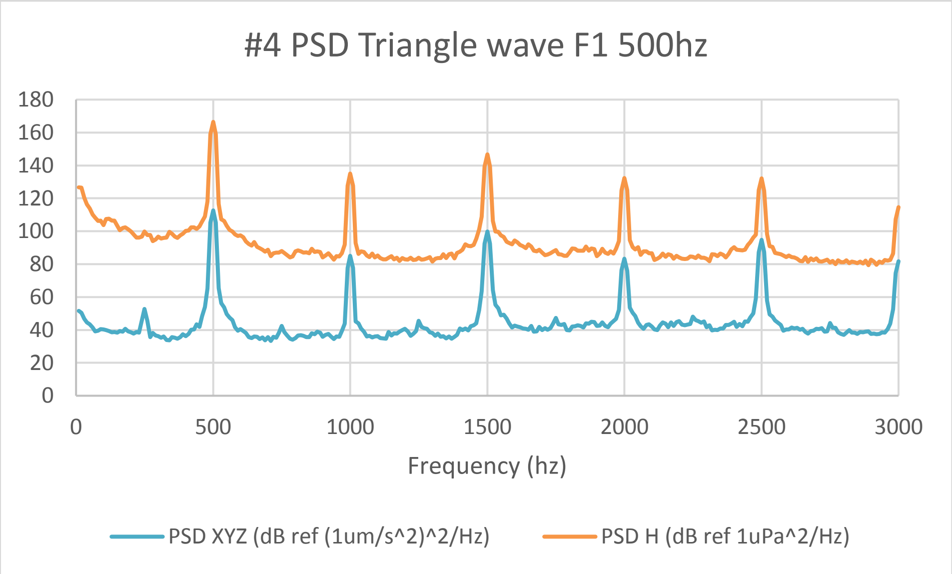
PSD H (dB ref 1uPa^2/Hz)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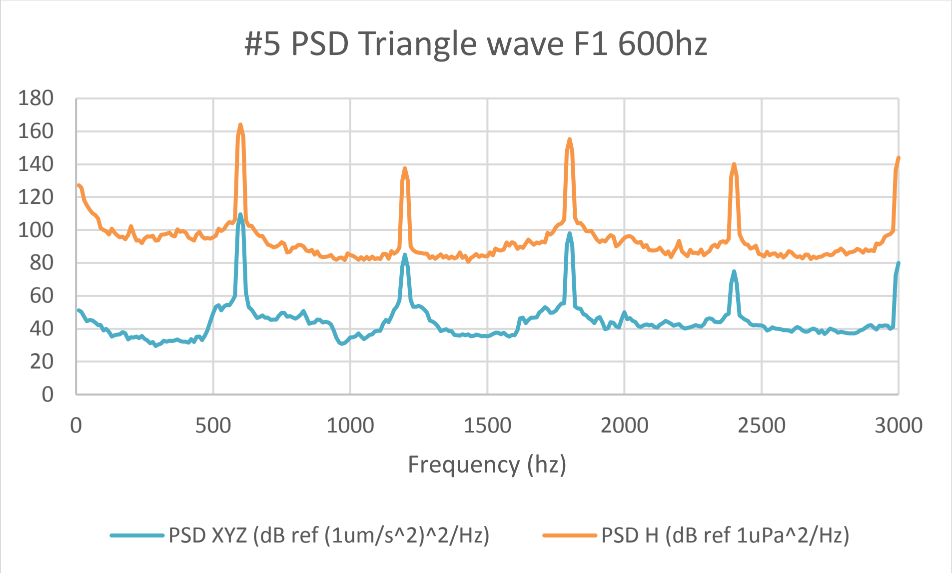
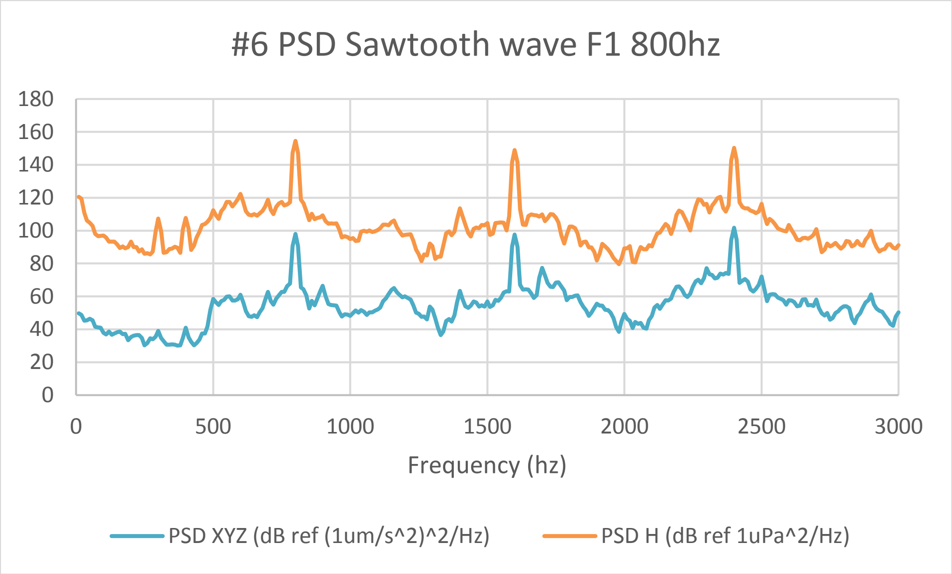
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#5 PSD Triangle wave F1 600hz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)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160
140
120
100
80
60
40
20
0
0
500
1000
1500
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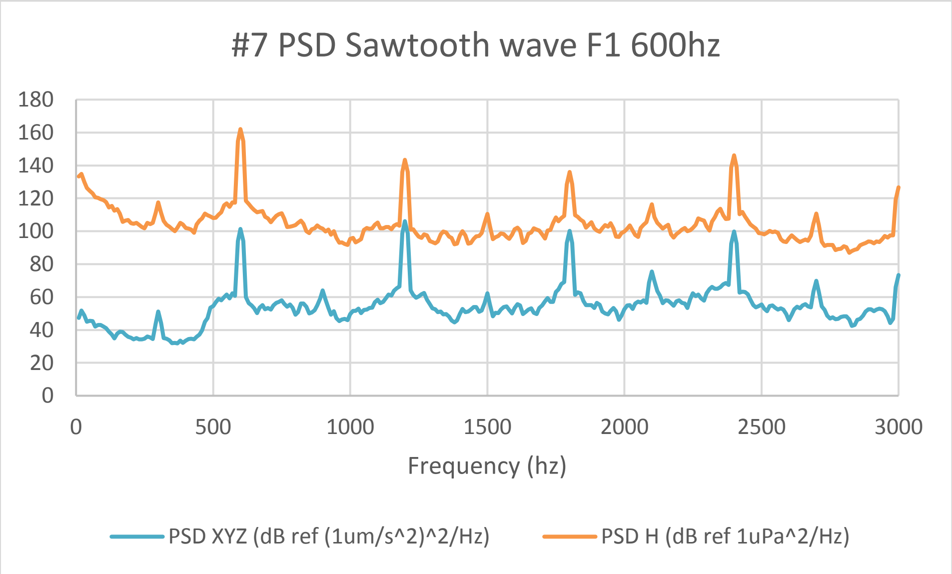
PSD H (dB ref 1uPa^2/Hz)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180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
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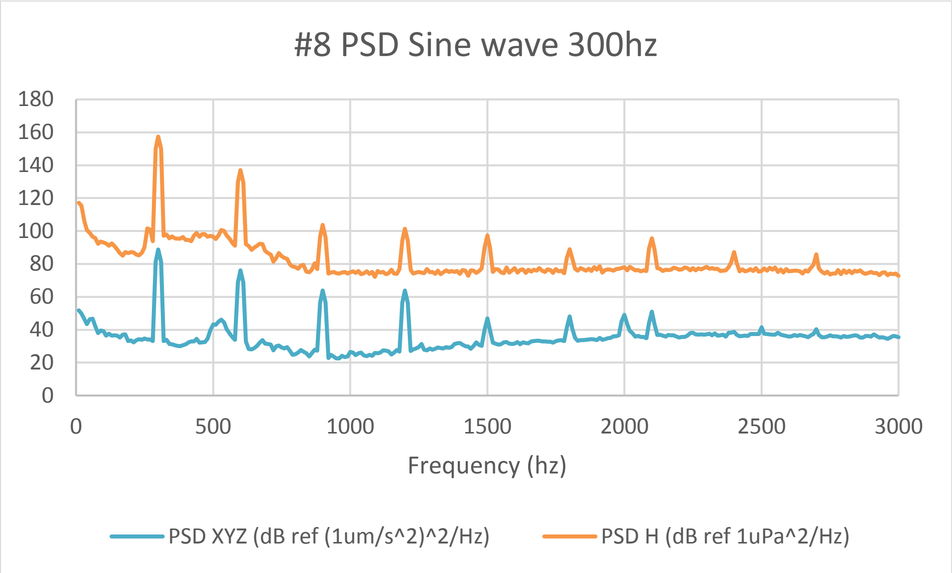
PSD H (dB ref 1uPa^2/Hz)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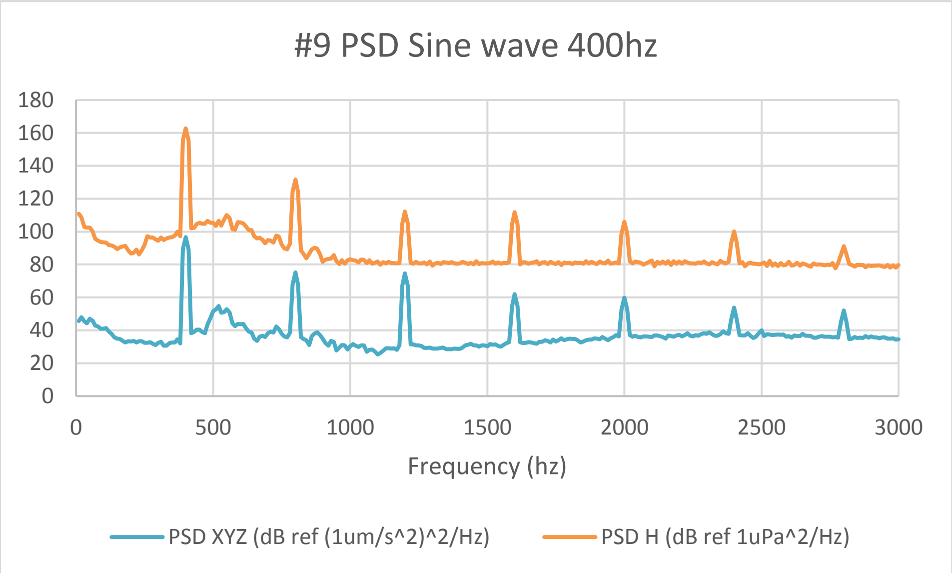
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)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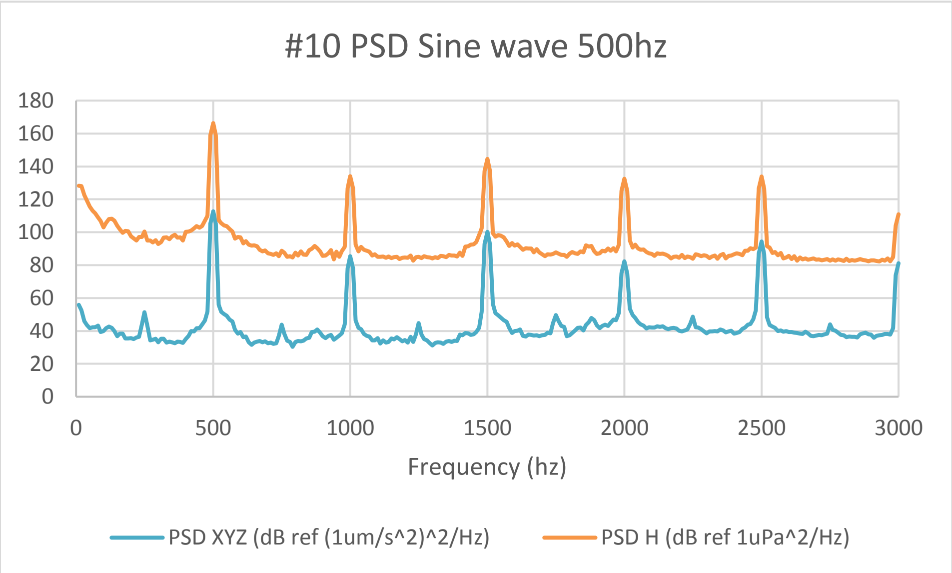
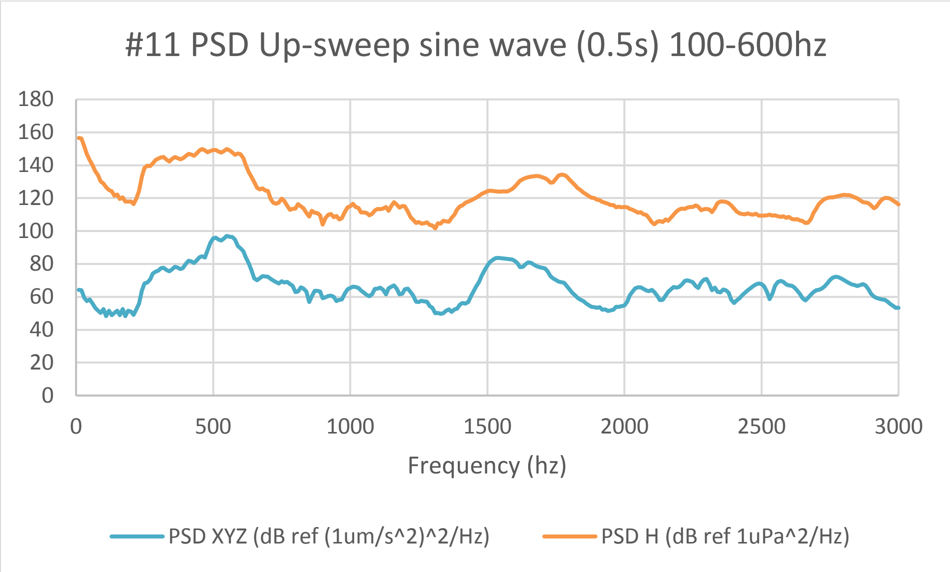
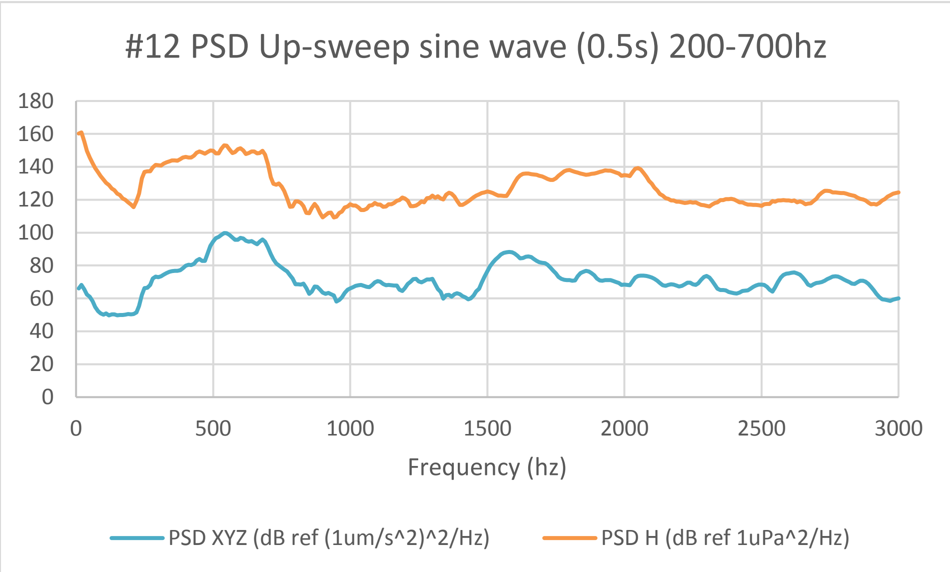
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#10 PSD Sine wave 500hz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)



#11 PSD Up-sweep sine wave (0.5s) 100-600hz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)



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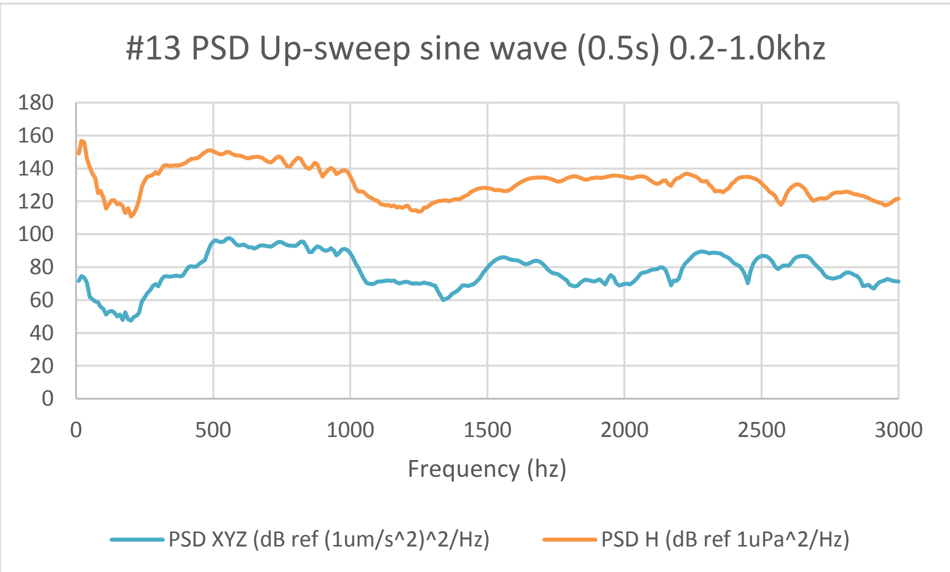
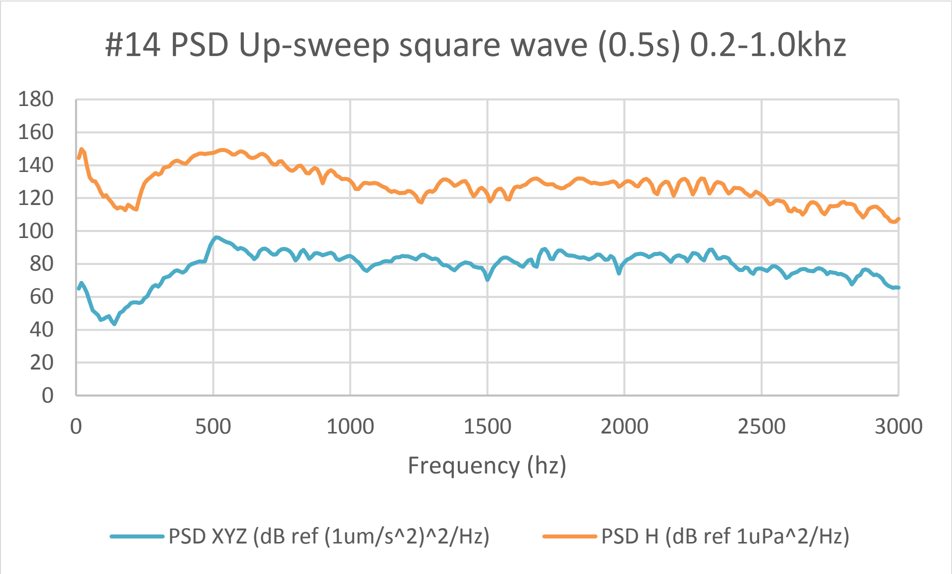
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)



#13 PSD Up-sweep sine wave (0.5s) 0.2-1.0khz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#14 PSD Up-sweep square wave (0.5s) 0.2-1.0khz
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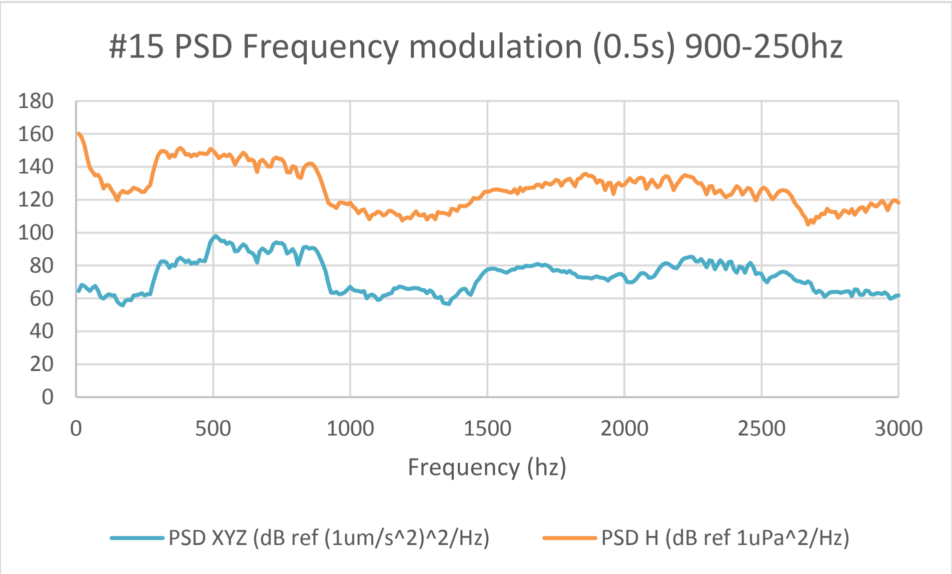
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#15 PSD Frequency modulation (0.5s) 900-250hz
180
160
140
120
100
80
60
40
20
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0
500
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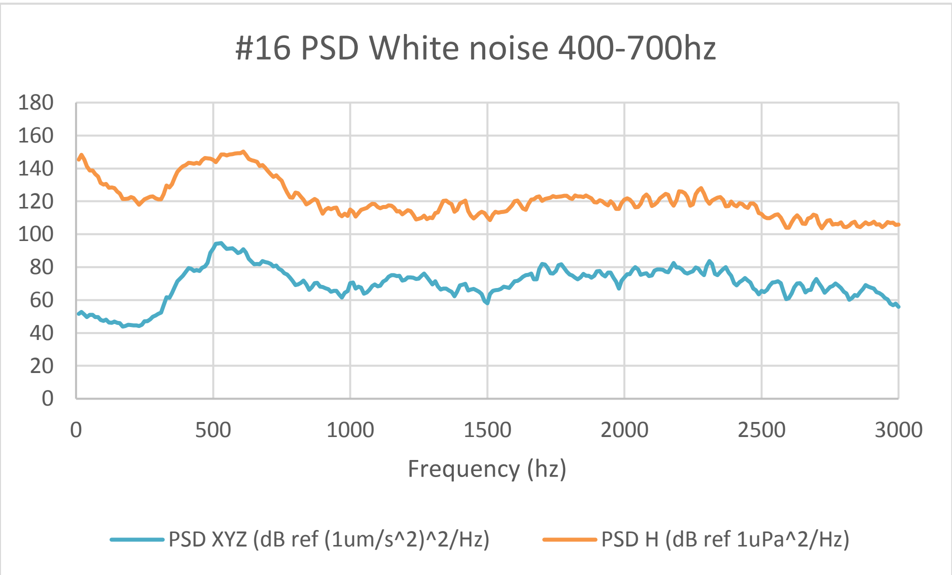
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)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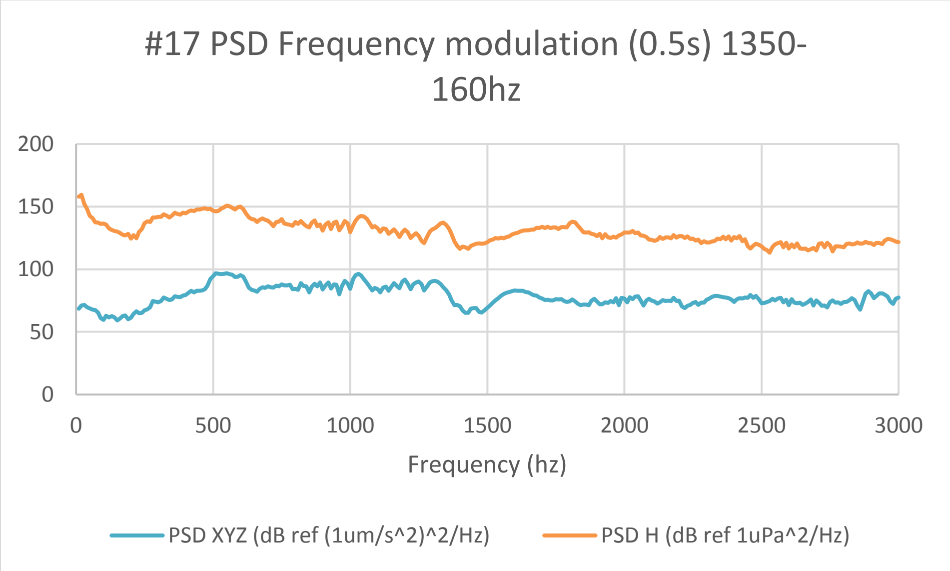
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)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150
100
50
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
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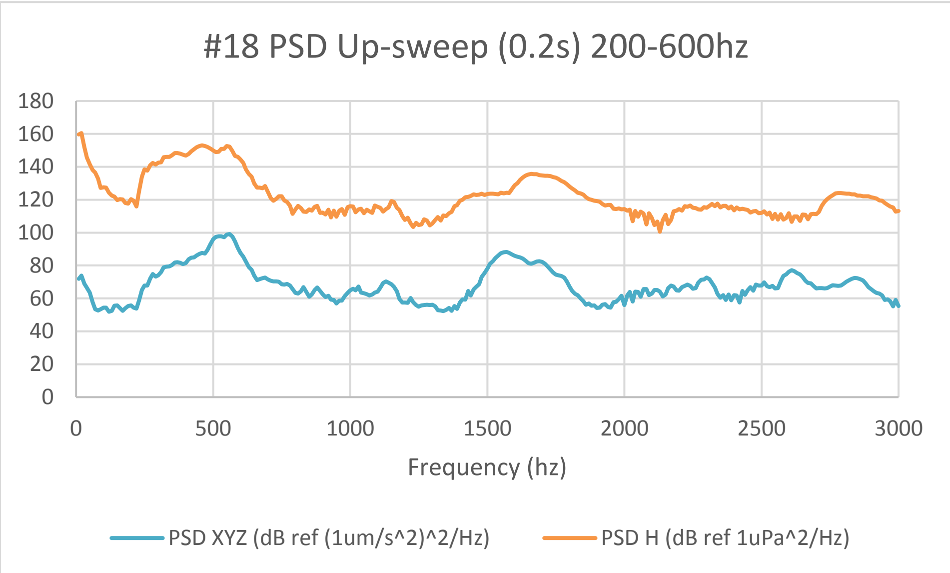
PSD H (dB ref 1uPa^2/Hz)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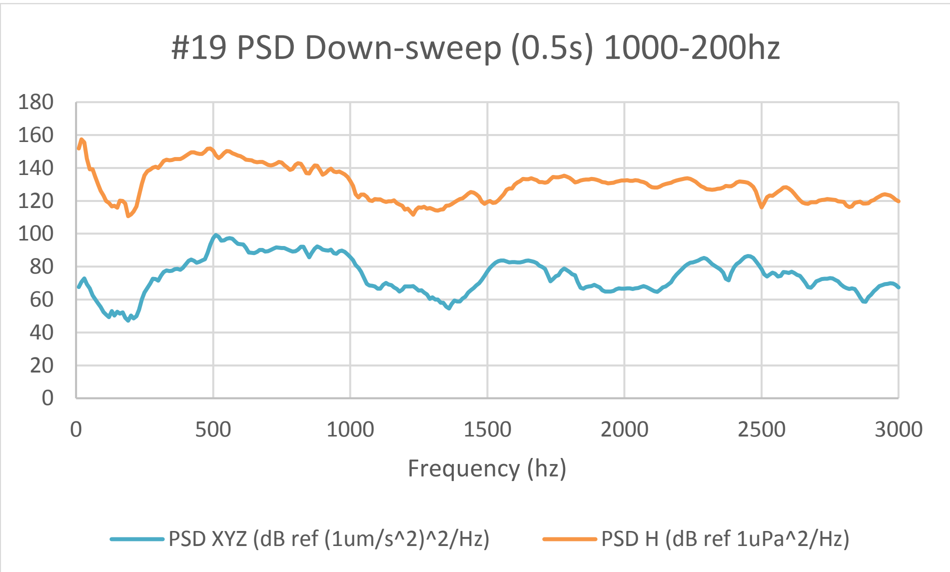
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)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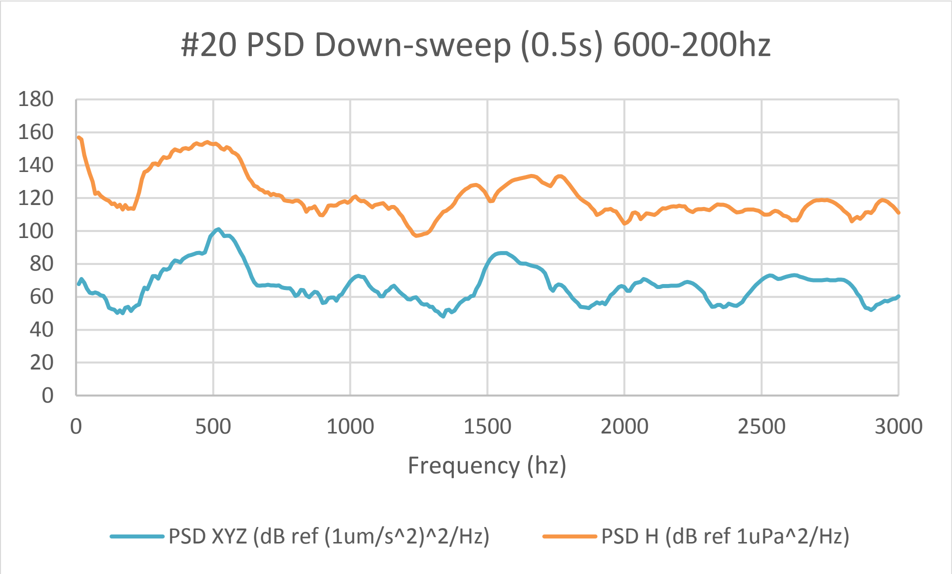
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)


#20 PSD Down-sweep (0.5s) 600-200hz
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
Frequency (hz)
PSD XYZ (dB ref (1um/s^2)^2/Hz)
PSD H (dB ref 1uPa^2/Hz)