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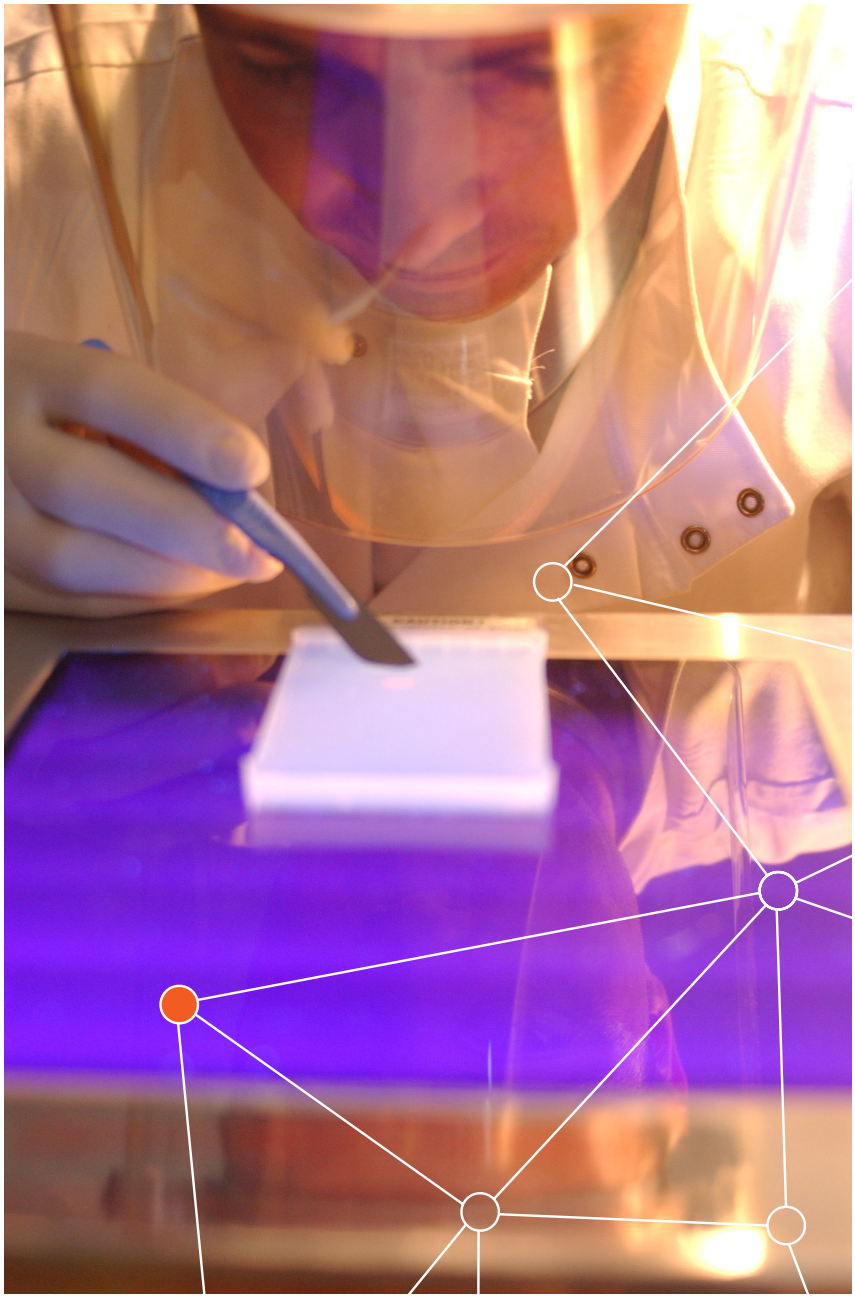

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Supporting variables for biological effects measurements in fish and blue mussel

T. Hansson • J. Thain • C. Martínez-Gómez • K. Hylland • M. Gubbins • L. Balk

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International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

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Correspondence about the details of any method or procedure should be directed to the author(s).

This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and demonstrated to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

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Contents

Abstract

Biological effects measurements in fish and blue mussel are fundamental in marine environmental monitoring. Nevertheless, currently used biomarkers may be confounded by basic physiological phenomena, such as growth, reproduction, and feeding, as well as thereby associated physiological variation. Here, we present a number of supporting variables, which are essential to measure in order to obtain reliable biological effects data, facilitate their interpretation, and make valid comparisons. For fish, these variables include: body weight, body length, condition, gonad maturation status, various somatic indices, age, and growth. For blue mussels, these variables include: volume, flesh weight, shell weight, and condition. Also, grossly visible anomalies, lesions, and parasites should be recorded for both fish and blue mussels. General confounding factors and their effects are described, as well as recommendations for how to handle them.

Keywords: age, biomarker, condition, growth, somatic index

1 Introduction

For all biological effects measurements within the Oslo-Paris Commission (OSPAR) Monitoring Programme, there is a requirement to report supporting biological parameters. For fish, these parameters include (Anonymous, 2008):

- species,
- sex,
- grossly visible anomalies, lesions and parasites,
- body weight,
- body length,
- gonad maturation status,
- gonadosomatic index (GSI),
- liver somatic index (LSI),
- age.

For shellfish (including blue mussels), these parameters include (Anonymous, 2012):

- body length,
- shell dry weight,
- soft tissue wet weight,
- condition index.

For both fish and blue mussels, the supporting parameters shall be reported in order to demonstrate that the investigated material is homogeneous and not confounded by more variation than necessary. The parameter values also determine to what extent the material is comparable with material from other investigations. A common alternative, however, is to accept a certain variation in the supporting biological characteristics listed by OSPAR. These are then used as supporting variables instead. The difference is that parameters are controlled by the investigator, whereas variables are allowed to vary and can be measured. The supporting variables may be used for statistical treatment of the confounding effect of certain variation. Some of them may also be measured as endpoints in their own right as general measures of health status. The measurement of organ sizes is very easy and can be performed with minimal instruction and equipment. The measurements should, however, always be performed and reported in a consistent and well-defined way. In many species, organ sizes can also be measured in material that has been stored frozen, provided that freeze drying during storage has been avoided. Here, we present a number of useful supporting variables which facilitate the interpretation of biological data generated within marine environmental monitoring.

2 Supporting metrics for fish

2.1 Body and growth characteristics

When measuring body metrics in fish, it is always necessary to consider the body size and development stage, as well as the reproductive cycle. In general terms, adults are sexually mature, whereas juveniles are sexually immature. The gonad may be referred to as immature or more or less mature, depending on the stage in the reproductive cycle. Generally, adult fish grow isometrically, i.e. the proportions of the body and its various parts remain the same when the overall size increases, whereas juvenile fish often grow allometrically, i.e. the proportions of the body and its various parts change when the overall size increases. The isometric growth of adult fish makes it possible to compare organ sizes between specimens of different body sizes. This is achieved by relating the organ size to an allometric standard, i.e. a part of the body whose size is not confounded by the phenomenon under study or any other confounding phenomenon. In juvenile fish, it is often impossible to make reliable comparisons of organ sizes between specimens of different body sizes. The only solution is to use specimens of a specific size class and/or development stage.

In many fish species, adults go through a natural annual reproductive cycle, which strongly affects their physiology and, as a consequence, many biomarkers. The most conspicuous phenomenon is the production of a gonad, whose content is released at spawning. At maturity, the gonad may constitute a substantial part of the body, especially in females. Less conspicuous, but equally important, is the annual cycle of the liver, whose size also changes over the year. In females, one function of the liver is to produce vitellogenin for incorporation in the eggs, and the intensity of this activity is related to the maturation of the eggs (Scott and Hylland, 2002). The reproductive cycle also involves variation in hormone status over the year. For example, the female gonadal cycle is paralleled by an oestrogen cycle, which affects e.g. the specific ethoxyresorufin-*O*-deethylase (EROD) activity (Gräns *et al*., 2010), which is a classical enzymatic biomarker for certain types of environmental pollution (Foureman *et al*., 1983; Balk, 1985; Stagg *et al*., 2016).

2.1.1 Body size

Body size is most commonly measured as body weight and body length. Body weight is usually measured as total weight including stomach and gut contents. If, however, the stomach is full of undigested food items that can be easily removed, these need to be excluded from the total weight. Sometimes the whole alimentary tract is excluded from the body weight, which may then be referred to as gutted body weight. Hence, it should always be specified how the body weight is measured. The recommended way to eliminate the variation in body weight due to variation in gonad weight is to calculate the somatic weight by subtracting the gonad weight from the body weight (Eqn. 1).

$$W_{somatic} = W_{body} - W_{gonad} \quad (1)$$

Here, "somatic" is the opposite of "reproductive" with respect to organs and tissues. It is commonly assumed that the somatic weight is much less variable than the gonad weight. There are three common ways to measure body length: standard length, fork length, and total length (Carlander and Smith Jr., 1945). Standard length is measured from the tip of the snout to the end of the body, exclusive of the caudal fin. Fork length is measured from the tip of the snout to the centre of the fork of the caudal fin. Total length is measured from the tip of the snout to the end of the caudal fin, either with the

caudal fin lobes spread out as *in vivo*, or with the lobes squeezed together. In the investigation by Carlander and Smith Jr. (1945), there were no significant differences in the correlation between the three length measures and body weight, whereas total length was possible to measure somewhat more accurately than the two other lengths. An advantage of total length is that it can be measured in all species, whereas not all species have a fork or a distinct beginning of the caudal fin. An advantage of standard length is that it is not confounded by fin erosion.

2.1.2 Somatic indices

The gonad size is an important indicator of the reproductive process. In adult fish, a common way to analyse gonad size is to calculate the gonadosomatic index (GSI), where the somatic weight is used as an allometric standard. GSI is the gonad weight expressed as percent of the somatic weight. Similarly, the liver somatic index (LSI) is the liver weight expressed as percent of the somatic weight. In the same way other somatic indices may be defined, e.g. spleen somatic index (SSI), visceral fat somatic index (VFSI), and heart somatic index (HSI). These types of somatic indices are valid as long as the somatic weight is not confounded by the phenomenon under study or any other confounding phenomenon. If the somatic weight is confounded, other allometric standards have to be sought. For example, the brain weight may be a good alternative, because the brain is one of the highest prioritized organs with respect to both homeostasis (Butterworth *et al*., 1986) and size (Xu *et al*., 2002). It is, perhaps, the last body function to be affected by noxious agents in many cases. Hence, the brain size is probably one of the best indicators of the structural ("real") size of the fish. Although only GSI and LSI are recommended within the OSPAR Monitoring Programme, all of the above indices may be useful. We have also used the brain as an allometric standard with very good results (e.g. Balk *et al*., 2016).

2.1.3 Sex

In some species, the sexes differ in external morphological characteristics, whereas in others they do not. Even in the latter type of species, sex determination is usually easy when mature (or maturing) gonads are present, whereas it may be impossible when the gonads are immature. Gonads may be paired or single. In perch (*Perca fluviatilis*), for example, the male gonad is paired, whereas the female gonad is single. In such species, sex determination is possible also in sexually immature specimens. In juveniles of many species, however, the sexes are often physiologically similar, rendering sex determination neither possible nor necessary.

2.1.4 Age and growth

Age is determined by analysis of natural growth lines on the scales, otoliths, vertebrae, fin spines, eye lenses, teeth, or bones of the jaw, pectoral girdle, and opercular series (Helfman *et al*., 2009). Otoliths are most widely used for marine fish and recommended when possible. Age may be used in the analysis of growth. It may also be a confounding variable for fish diseases, which often are more prevalent in older fish (Stentiford *et al*., 2010).

2.1.5 Other morphological characteristics

Other morphological characteristics that may be of interest include lesions and pigmentation changes, both in external organs, such as skin, fins, and eyes, and internal organs, such as liver and gonad. For example, a variety of gonadal abnormalities have been observed in herring (*Clupea harengus*) in the northern Baltic Sea during the last

decades (Rajasilta *et al*., 2016). A commonly occurring lesion in polluted waters is fin erosion (e.g. Noaksson *et al*., 2005b; Hansson *et al*., 2014a). There may also be decreased astaxanthin pigmentation of the fins and ovary and/or increased melanin pigmentation of the liver and ovary. Such morphological characteristics can easily be documented with modern digital camera technology. The NCS Natural Color System©® may be used for standardized colour measurements, which may also be converted to quantitative measures of colour (Balk *et al*., 2009). To facilitate analysis of outer morphological characteristics, sampled specimens should be photographed on both sides before dissection. Inner morphological characteristics should also be photographed.

2.2 General confounding factors

General confounding factors which may affect the supporting variables include:

- species,
- sex,
- feeding preferences,
- feeding status,
- parasite infestation and other disease,
- location,
- season.

The confounding effects of species and sex are obvious, because of the many well-known physiological differences between species and the sexes. Feeding preferences may change as the fish grow and may thus compromise comparison of specimens of different sizes. Feeding status refers to the amount of gut contents. Parasite infestations may bias the measurement of organ weights, directly by constituting a significant part of the weight of an organ, and/or indirectly by affecting a specimen's physiology. Other diseases may also affect a specimen's physiology. For example, open ulcers may decrease the specific EROD activity in various organs (Hansson *et al*., 2014a). Different locations and seasons may result in differences in food, day length, light intensity, water temperature, and salinity, as well as other physicochemical differences (e.g. Dabrowski *et al*. 1996; Hansson *et al*., 2006a). Different fishing methods may differ in efficiency and be selective for specimens with a certain degree of swimming activity and/or a certain movement pattern.

It is recommended to keep the variation in all potential confounding factors to a minimum, both within and between the investigated groups. Ideally, toxic effects should be measured in both sexes, especially when investigating endocrine disruption, but if this is not possible, a way to eliminate the influence of sex is to use only one of the sexes. Different feeding preferences may be avoided by restricting the size range to specimens with the same feeding preferences. The confounding effect of feeding status may be eliminated by keeping the fish in a corf (well protected from disturbances) at the capture site for a few days for defecation. This also has the advantage that the fish recover from stress induced by the capture and handling, and that bile accumulates in the gall bladder. If specimens with disease or parasites are rare in the investigated material, they may be excluded from the analysis. If they are more frequent, however, they should be analysed as a separate group. Depending on the particular variables investigated and the particular disease, it may also be appropriate to analyse the degree of disease. It is, however, very difficult to give general advice on how this should be done. It is also informative to compare the frequency of parasites between different exposure groups. Even when specimens with disease or parasites are rare, their presence should be recorded, e.g. to provide a record of when they were first observed. The

confounding effects of location and season may be eliminated by using a local control station, i.e. relatively close to the other stations, and by sampling at the same time of the year. The fishing should be performed in a consistent way for all investigated groups. Generally, many errors will cancel each other out if the sampling and measurements are performed in a consistent way.

2.3 Condition

A commonly used measure of fish condition is Fulton's condition factor (CF) (Nash *et al*., 2006). It is defined as the weight (W) of the fish divided by its cubed length (L), and usually a scaling factor (f) is applied to bring the condition factor close to 1 (Eqn. 2).

$$CF = \frac{W}{L^3} \cdot f \tag{2}$$

Fulton's condition factor is constant in specimens of equal shape and density (e.g. specimens that grow isometrically), and it is higher in fat specimens than in lean specimens. It is obvious that this condition factor is species dependent, because of natural differences in shape and density between fish species. Fulton's condition factor is also referred to as condition index (CI). Often, when the CF or CI are based on the somatic body weight, this is emphasized by using the terms somatic condition factor (SCF) or somatic condition index (SCI). Although Fulton's condition factor has been used for most different fish species, it is inappropriate for fish with a very different morphology, e.g. flatfish, or in cases of allometric growth. In such cases, it is advisable to use the relative condition factor (Eqn. 3), which is comprehensively described by Froese (2006).

$$CF_{rel} = \frac{W}{a \cdot L^b} \tag{3}$$

Weight and length are measured for each individual, and the constants a and b are estimated by a log-log regression on the relationship between weight and length (Eqn. 4) in a reference material.

$$\log(W) = \log(a) + b \cdot \log(L) \tag{4}$$

Only if b does not differ significantly between groups is it appropriate to compare the relative condition factor of different groups (Froese, 2006).

The use of measures of condition in investigations of adverse effects of xenobiotics has been reviewed by van der Oost *et al*. (2003). Condition has been analysed more often in long-term field investigations than in short-term exposure laboratory experiments, because it responds relatively slowly to various conditions. Recent investigations have shown that condition may be decreased by diseases (Anonymous, 2011). There are, however, examples where impaired reproduction has been related to increased condition (Noaksson *et al*., 2003a). The most likely explanation of this latter observation is that more resources were allocated to somatic growth, when no gametes were produced.

2.4 Gonad maturation status

In healthy adult fish that spawns every year, the gonad has a natural annual cycle. This has been demonstrated in e.g. female perch by Noaksson *et al*. (2004). It has also been demonstrated that healthy female perch that are adult by size should produce eggs

every year. This concept is based on investigations of perch from the 1950s, when the environment as a whole was less affected by human activities than it is today. In the extensive works by Le Cren (1951, 1958) and Alm (1959), all female perch longer than 19 cm (total length) developed eggs. Hence, the more recent occurrence of adult fish that do not produce a gonad must be considered as a serious disturbance, and therefore it is informative to analyse the frequency of this phenomenon. Especially in perch, adults without a maturing gonad have been denoted SIM (sexually immature) as opposed to SM (sexually mature).

The ultimate cause of the SIM phenomenon among adults is unknown but it has been linked to toxic exposure. Increased frequency of SIM females has been observed for perch, roach (*Rutilus rutilus*), and brook trout (*Salvelinus fontinalis*) exposed to leachate from refuse dumps (Noaksson *et al*., 2001, 2003a, 2004, 2005a) and for Atlantic croaker (*Micropogonias undulatus*) exposed to naphthalene and water soluble fractions of diesel fuel oil in the laboratory (Thomas and Budiantara, 1995).

Field investigations where a delay or lack of gonad development has been observed include: burbot (*Lota lota*) at the northern coast of the Bothnian bay, Finland and Sweden (Pulliainen *et al*., 1992); English sole (*Parophrys vetulus*) in generally polluted areas in Puget sound, WA, USA (Johnson *et al*., 1988); and perch in the recipients of pulp and paper mills in Sweden (Sandström *et al*., 1988; Sandström, 1994; Hansson *et al*., 2014a) and white sucker (*Catostomus commersonii*) in such recipients in Ontario, Canada (McMaster *et al*., 1991).

In female perch from background areas, adult SIM specimens occur only in more recent (last 2–3 decades) investigations (Sandström, 1994; Sandström *et al*., 1995; Lukšienė *et al*., 2000; Noaksson *et al*., 2001, 2004, 2005a; Sandström and Neuman, 2003; Roots *et al*., 2004; Hansson *et al*., 2006a; Linderoth *et al*., 2006).

In various investigated groups of adult female perch, increased SIM frequency was quantitatively related to decreased GSI in SM specimens (Hansson *et al*., 2006a; Linderoth *et al*., 2006).

2.5 Gonadosomatic index (GSI)

Reproduction is one of the most important processes in living organisms and there are numerous examples of how it can be disturbed by toxic substances. There is a wide range of toxic effects, from simple cell or tissue death to the disruption of sophisticated mechanisms of endocrine regulation. A reproductive disturbance may lead to an altered number of eggs, altered size of the eggs, a temporal shift in the reproductive cycle, or a combination thereof. Such toxicity may also have severe effects on population sizes and genetic diversity. GSI is the gonad weight expressed as percent of the somatic weight (Eqn. 5), and in adults of many fish species it has a natural annual cycle.

$$GSI = 100 \cdot \frac{W_{gonad}}{W_{somatic}} \tag{5}$$

Group comparisons must take the natural variation in GSI into account, and the requirement for an appropriate control group cannot be overemphasized. For example, GSI in SM female perch in Sweden has been demonstrated to increase almost linearly at a rate of ca 0.08 percentage units per day during September through November (Noaksson *et al*., 2004). Such growth rates of the gonad may cause significant differences in GSI within a few weeks (e.g. Noaksson *et al*., 2005b). It is also generally recommended to avoid sampling during spawning, because of the drastic changes in both

GSI and hormone status during this period. Baseline investigations may be used to find suitable times for the analysis of GSI (Förlin and Haux, 1990; Larsen *et al*., 1992; Noaksson *et al*., 2004).

GSI has been widely used as a biomarker in fish, both in field investigations and laboratory experiments (Kime, 1995). Decreased GSI has been observed in fish exposed in the field to bleached pulp and paper mill effluents (Andersson *et al*., 1988; Sandström *et al*., 1988; McMaster *et al*., 1991; Balk *et al*., 1993; Förlin *et al*., 1995; Hansson *et al*., 2014a), as well as chlorine-free pulp and paper mill effluents (Karels *et al*., 2001), or general pollution (Johnson *et al*., 1988; Noaksson *et al*., 2001, 2003a; Linderoth *et al*., 2006; Hansson *et al*., 2014a).

Decreased GSI has also been observed in fish exposed at the laboratory to petroleum mixtures (Truscott *et al*., 1983; Kiceniuk and Khan, 1987), specific polycyclic aromatic hydrocarbons (PAHs) (Thomas, 1988; Singh, 1989; Thomas and Budiantara, 1995), the polychlorinated biphenyl (PCB) mixture Aroclor 1254 (Thomas, 1988), pesticides (Ram and Sathyanesan, 1986; Singh, 1989), lead (Thomas, 1988), and cadmium (Singh, 1989).

2.6 Liver somatic index (LSI)

The liver is responsible for many important functions in animals, such as carbohydrate, protein, lipid, and hormone metabolism and storage. It is also active in detoxification and the immune defence. There are numerous examples of how liver size and function can be altered by toxic substances. LSI is the liver weight expressed as percent of the somatic weight (Eqn. 6), and it is sometimes also called hepatosomatic index (HSI).

$$LSI = 100 \cdot \frac{W_{liver}}{W_{somatic}} \tag{6}$$

In adult fish, LSI may have a natural annual cycle, which may be more pronounced in females than in males, owing to production of vitellogenin for incorporation in the maturing eggs. Toxic exposure may lead to either increased LSI by hyperplasia and/or hypertrophy, or decreased LSI by loss of cells (by apoptosis and/or necrosis) or cell atrophy. It should be noted that changes in LSI also affect the total liver detoxification capacity, for example the cytochrome P450 1A (CYP1A) activity, commonly measured as EROD activity. An attempt to quantify the total liver EROD activity was made by Hansson *et al*. (2006b), who defined a liver EROD somatic index (EROD-SI) as the total liver EROD activity per kilogram somatic weight. Another common phenomenon is that LSI is decreased in cases of particularly severe toxic exposure, where other biomarkers have passed their normal dose-response range (e.g. Hansson *et al*., 2006b, 2014b; Linderoth *et al*., 2006). LSI may thus be altered both by specific substances and by generally compromised health due to toxic exposure.

Group comparisons must take the natural variation in LSI into account, and the requirement for an appropriate control group cannot be overemphasized. For example, LSI in adult female perch in Sweden has been demonstrated to increase almost linearly at a rate of ca 0.01 percentage units per day during September through November (Noaksson *et al*., 2004). Such growth of the liver may cause significant differences in LSI within a few weeks (e.g. Noaksson *et al*., 2005b). Baseline investigations may be used to find suitable times for the analysis of LSI (Förlin and Haux, 1990; George *et al*., 1990; Larsen *et al*., 1992; Noaksson *et al*., 2004). An example of the utility of such baseline investigations is given by Noaksson *et al*. (2004), who demonstrated that there was no obvious difference in LSI between SM and SIM female perch over the year. Hence, the observation of significantly lower LSI in SIM females than in SM females in other

investigations (e.g. Linderoth *et al*., 2006) may be interpreted as parallel expressions of compromised health, i.e. both disturbed reproduction (in the SIM females) and decreased LSI in the same individuals.

LSI has been established as a biomarker in fish by several investigations (van der Oost *et al*., 2003). Altered LSI has been observed in fish exposed to pulp and paper mill effluents in the field. In such investigations, LSI was either increased (Andersson *et al*., 1988; Lehtinen *et al*., 1990; Hodson *et al*., 1992; Kloepper-Sams and Owens, 1993; Förlin *et al*., 1995; Huuskonen and Lindström-Seppä, 1995) or decreased (Balk *et al*., 1993; Förlin *et al*., 1995). The opposite LSI responses were probably due to differences in dose and/or chemical composition of the pulp and paper mill effluents.

Other complex mixtures that have been demonstrated to alter LSI include leachate from public refuse dumps (Noaksson *et al*., 2001, 2003a) and effluents from sewage-treatment plants (Kosmala *et al*., 1998).

Increased LSI by exposure to organochlorines or PAHs in the field has been demonstrated in several investigations (Slooff *et al*., 1983; Goksøyr *et al*., 1991; Leadley *et al*., 1998; Kirby *et al*., 1999a, 1999b; Stephensen *et al*., 2000).

Altered LSI has also been observed in fish exposed at the laboratory to organochlorines (Adams *et al*., 1990; Newsted and Giesy, 1993; Arnold *et al*., 1995; Otto and Moon, 1995; Gadagbui and Goksøyr, 1996; Åkerblom *et al*., 2000), PAHs (Singh, 1989; Celander *et al*., 1994), decabromodiphenyl ether (Kierkegaared *et al*., 1999), two-stroke outboard engine exhausts (Tjärnlund *et al*., 1996), pesticides (Singh, 1989; Åkerman *et al*., 2003), and cadmium (Singh, 1989; Pereira *et al*., 1993).

2.7 Other somatic indices

As mentioned above, other somatic indices may be defined by expressing an organ weight as percent of the somatic weight. Examples of such somatic indices include i.a. spleen somatic index (SSI), visceral fat somatic index (VFSI), and heart somatic index (HSI). In fish, the spleen produces and stores red blood cells. Acute stress may lead to rapid release of red blood cells from the spleen to the circulatory system (Fänge and Nilsson, 1985), which may result in decreased spleen size (Yamamoto, 1989). SSI has been used as a biomarker in a limited number of investigations (Kiceniuk and Khan, 1987; Ericson *et al*., 1998; Linderoth *et al*., 2006), but its significance needs further analysis, since both an increase and a decrease of SSI seem to be possible results of toxic exposure. Visceral fat is often present around the intestine, and may vary in abundance depending on health status. The biomarker potential of VFSI has been demonstrated in a limited number of investigations (McMaster *et al*., 1991; Linderoth *et al*., 2006; Karami *et al*., 2011), but it may be difficult to analyse with the most common statistical methods, since data are not always normally distributed. For example, in the investigation by Linderoth *et al*. (2006), control specimens had a moderate amount of visceral fat, whereas many of the unhealthiest specimens had either very much or very little visceral fat. HSI has been analysed in some investigations (Kiceniuk and Khan, 1987; Lennquist *et al*., 2011), but its significance needs further evaluation.

2.8 Growth

In order to analyse growth, it is necessary to know the age of the fish. In its simplest form, growth may be analysed by dividing the somatic weight by the age (Noaksson *et al*., 2001, 2003a, 2003b, 2005b). This measure is often called somatic growth (SG). Generally, however, SG will be confounded by the size of the fish, because the yearly

weight increments increase as the fish grows. This problem may be resolved by including body length as a covariate in the statistical analysis and reporting the results as predicted values for the average length of the investigated specimens. This procedure was successfully applied e.g. in the investigation by Hansson *et al*., (2014a). Another way to analyse growth is so called back-calculated growth, where the thickness of the natural growth lines of e.g. a scale is related to body length (Bagenal, 1978). This technique requires that a (species specific) standard curve is constructed by analysis of a reference material of specimens of different ages. Back-calculated growth yields a growth curve for each investigated specimen, whereas SG represents the average growth of a specimen over time. Statistically, SG may be easier to compare between exposure groups than back-calculated growth.

Retarded growth due to toxic exposure has been demonstrated in several investigations (e.g. McMaster *et al*., 1991; Ericson *et al*., 1998; Noaksson *et al*., 2003a, 2005b; Couillard *et al*., 2005; Linderoth *et al*., 2006; Hansson *et al*., 2014a). Hence, the occurrence of many small individuals in an area does not necessarily indicate good reproduction. A good example of how age, size, and condition of the fish significantly confounded a more advanced biomarker, specific liver EROD activity, is given by Couillard *et al*. (2004).

3 Supporting metrics for the blue mussel

3.1 Systematics

Blue mussels consist of at least three closely related taxa known as the *Mytilus edulis* complex (Väinölä and Hvilsom, 1991). The main component taxa are *M. edulis*, *M. trossulus*, and *M. galloprovincialis*. Collectively, they populate both coasts of the North Atlantic, including the Baltic Sea, as well as other parts of the world (Seed, 1992). The component taxa have been shown to hybridize with each other when present at the same locality (Koehn *et al*., 1984; Väinölä and Hvilsom, 1991; Väinölä and Strelkov, 2011). For example, the Baltic Sea and the Swedish west coast are populated by both *M. edulis* and *M. trossulus* as well as hybrids of the two (Väinölä and Strelkov, 2011). Hybridization has recently been facilitated by human activities, such as shipping (ballast water), whereby taxa have been introduced to new areas. Since the taxonomic relationships within the *Mytilus edulis* complex are still under scientific debate, we refer to the component taxa simply as blue mussels. A few investigations of the component taxa have studied differences in bioaccumulation and biological response to toxicity (Brooks *et al*., 2015). Some results indicate that such differences between taxa may occur, although this needs to be confirmed by further investigations (Brooks *et al*., 2015).

3.2 Body and growth characteristics

Generally, blue mussels that are large enough to be examined with the unaided eye grow isometrically, i.e. the proportions of the body and its various parts remain the same when the overall size increases. The isometric growth of blue mussels makes it possible to compare organ sizes between specimens of different body sizes. This is achieved by relating the organ size to an allometric standard, i.e. a part of the body whose size is not confounded by the phenomenon under study or any other confounding phenomenon. Blue mussels may spawn at different times at different places. For example, in northern Europe, blue mussels have their main spawning season in late winter to late spring, depending on latitude (Seed, 1976). This is important to consider when designing investigations and/or monitoring of geographically separated individuals. Blue mussels make a huge investment in their reproduction. From the onset of gametogenesis until spawning, the flesh weight may be doubled (e.g. Kautsky, 1982). The gonad is, however, produced partly at the expense of the somatic tissues. Hence, the flesh weight is at maximum just before spawning, and at minimum just after spawning (Mason, 1969; Seed, 1976).

3.3 General confounding factors

General confounding factors, which may affect the supporting variables include:

- species,
- sex,
- feeding preferences,
- feeding status,
- parasite infestation and other disease,
- exposure to air when situated in the intertidal zone
- location,
- season.

Sex determination is usually easy when mature (or maturing) gonads are present, whereas it may be impossible to determine sex outside of the reproductive period. Feeding preferences may change as the blue mussel grows, and may thus compromise

comparison of specimens of different sizes. Feeding status refers to the amount of gut contents. Parasite infestations and other diseases may bias the measurement of flesh weight (Kent, 1979; Theisen, 1987). Blue mussels situated in the intertidal zone are regularly exposed to air, which affects their physiology. Different locations and seasons may involve differences in food, day length, light intensity, water temperature, salinity, as well as other physicochemical differences.

A general recommendation is to keep the variation in all potential confounding factors to a minimum. It is our impression that the sexes are physiologically more similar in blue mussels than in fish, at least outside the reproductive period, so it may be acceptable to ignore the difference between the sexes, at least for certain biomarkers. Different feeding preferences may be avoided by restricting the size range to specimens with the same feeding preferences. The confounding effect of feeding status may be eliminated by allowing the blue mussels to defecate before sampling. Gut clearance is usually accomplished within 24 hours. If specimens with disease or parasites are rare in the investigated material, they may be excluded from the analysis. If they are more frequent, however, they should be analysed as a separate group. Depending on the particular variables investigated and the particular disease, it may also be appropriate to analyse the degree of disease. It is, however, very difficult to give general advice how this should be done. It is also informative to compare the frequency of parasites between different exposure groups. Even when specimens with disease or parasites are rare, their presence should be recorded, e.g. to provide a record of when they were first observed. Specimens regularly exposed to air and specimens constantly submerged should be analysed as separate groups. The confounding effects of location and season may be eliminated by using a local control station, i.e. relatively close to the other stations, and by sampling at the same time of the year. Generally, many errors will cancel each other out if the sampling and measurements are performed in a consistent way.

3.4 Condition

Many different condition indices have been used for blue mussels and there is no generally accepted standard (e.g. Davenport and Chen, 1987). Here, we discuss general aspects of blue mussel condition and different ways to measure the variables used for computation of various condition indices. Condition refers to flesh weight in relation to an allometric standard, which indicates the structural ("real") size of the blue mussel. The flesh weight may be determined for fresh, cooked, or dried flesh. Cooked or dried flesh weights have been frequently used for their convenience. Dried flesh weight is also independent of varying water content. If, however, biochemical or chemical analysis of the flesh is desired, fresh weight has to be used, because cells, organelles, enzymes, and certain nutrients are destroyed by cooking or drying. Allometric standards that have been used include: live weight (including internal water); outer or inner volume; shell length; and shell weight. The flesh dry weight proportion of the fresh weight has also been used as a measure of condition. Although blue mussels that are large enough to be examined with the unaided eye grow mainly isometrically, condition is partly confounded by body size (Baird, 1958).

Throughout the scientific literature, several different drying regimes have been used for determination of dried flesh weight. Drying has been performed at various temperatures ranging from 60 to 95 °C for 24 to 48 h, or until constant weight. Two of the more common drying regimes are 60 °C for 48 h and 95 °C for 24 h. In order to compare these two drying regimes a control experiment was performed, where the flesh of 30 blue mussels from the Baltic Sea was first dried at 60 °C for 48 h and weighed, and then dried at 95 °C for additional 24 h and weighed again (Balk *et al*., 2016). The 95 °C drying

regime yielded a 4.4% lower dry weight than the 60 °C drying regime. The difference is probably due to evaporation of crystal water at the higher temperature. Dried flesh weight may also be analysed as ash-free dry weight (e.g. Rodhouse *et al.*, 1984). We recommend drying at 95 °C for 24 h.

The outer volume may be calculated as the product of specimen length, width, and height, and an empirically determined constant, which may be calculated from the total weight of specimens completely filled with water at the beginning of the sampling, taking into account shell wet weight and density, water density, and soft body wet density. The inner volume may be determined by filling the cleaned valves with sand with known density and then weighing the sand (Aldrich and Crowley, 1986). The shell weight may be either wet or dry. We recommend the outer volume (V), determined by measuring length, width, and height of the intact mussel, as the allometric standard for calculation of condition (C), both when fresh and dried flesh weight (W) are used (Eqn. 7).

$$C = \frac{W_{flesh}}{V_{outer}} \tag{7}$$

3.5 Other indices

Other indices that have been suggested for blue mussels include shell condition index (SCI), digestive gland condition index (DGCI), and digestive gland body index (DGBI) (Tomas Hansson, unpublished data). SCI is the shell wet weight (exclusive of large fouling organisms such as barnacles) divided by the outer volume (Eqn. 8).

$$SCI = \frac{W_{shell}}{V_{outer}} \tag{8}$$

SCI is, however, often confounded by small fouling organisms, such as moss animals, which are laborious to remove. A solution could be to quantitate such fouling, e.g. as the proportion of fouled shell surface area. The relationship between fouling and SCI can then be determined and used to normalize SCI with respect to fouling (Tomas Hansson, unpublished data). The digestive gland (hepatopancreas) corresponds to the liver and pancreas in vertebrates. It is responsible for a large number of metabolic functions. DGCI is the digestive gland wet weight divided by the outer volume (Eqn. 9), whereas DGBI is the digestive gland wet weight expressed as percent of the (total) wet flesh weight (Eqn. 10).

$$DGCI = \frac{W_{DG}}{V_{outer}} \tag{9}$$

$$DGBI = 100 \cdot \frac{W_{DG}}{W_{flesh}} \tag{10}$$

4 Conclusions

Although many biomarkers have been subject to various types of quality assurance, the supporting variables presented here are essential to measure in order to obtain reliable biological effects data, facilitate their interpretation, and make valid comparisons. The way these supporting variables are measured needs to be reported in detail, for example whether total or gutted weight is used. A general recommendation is to keep the variation due to confounding factors to a minimum. Making the investigated material as homogeneous as possible with respect to confounding factors not only facilitates comparison between exposure groups, it also increases the statistical power (Gagnon and Hodson, 2012).

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Author contact information

Tomas Hansson
Department of Environmental Science and Analytical Chemistry (ACES)
Stockholm University
10691 Stockholm
Sweden
tomas.hansson@aces.su.se

John Thain
Centre for Environment Fisheries and Aquaculture Science (Cefas)
The Nothe, Barrack Road
Weymouth
Dorset DT4 8UB
United Kingdom
johnthain41@gmail.com

Concepción Martínez-Gómez
Instituto Español de Oceanografía
Centro Oceanográfico de Murcia
Varadero 1, San Pedro del Pinatar
30740 Murcia
Spain
concepcion.martinez@mu.ieo.es

Ketil Hylland
Department of Biosciences
University of Oslo
PO Box 1066 Blindern
0316 Oslo
Norway
ketil.hylland@ibv.uio.no

Matthew Gubbins
Marine Scotland – Marine Laboratory
375 Victoria Road
Aberdeen AB11 9DB
United Kingdom
matthew.gubbins@gov.scot

Lennart Balk
Department of Environmental Science and Analytical Chemistry (ACES)
Stockholm University
10691 Stockholm
Sweden
lennart.balk@aces.su.se