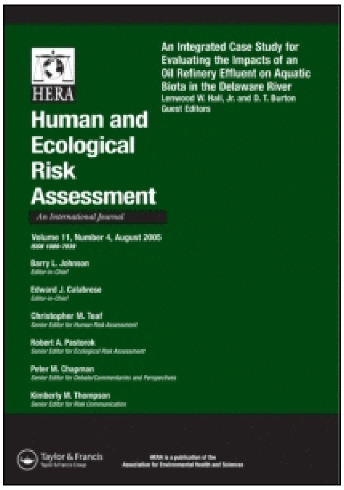

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Aquatic Behavioral Ecotoxicology—Prospects and Limitations

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ABSTRACT

This Perspective gives an overview of the definitions, advantages, and the importance of behavioral studies in ecotoxicology. Recent developments in automated quantitative recording technology as well as in mathematical data analysis and statistical data treatment have given rise to increased research in behavioral ecotoxicology. As an example, several studies performed with different invertebrate and vertebrate species using the non-optical impedance recorder, the Multispecies Freshwater Biomonitor$^{\text{TM}}$, are mentioned and discussed under the aspect of recent developments. However, some problems have still to be solved, such as (1) linking behavior to other biological test parameters on the suborganismal as well as the population level, (2) standardization of test designs and methods, and (3) creating more acceptance of these methods in the legislative framework of biomonitoring.

Key Words: behavior, biomarker, stress, biomonitor, ecotoxicology, Stepwise Stress Model, avoidance, Multispecies Freshwater Biomonitor$^{\text{TM}}$.

WHAT IS BEHAVIOR?

Behavior arises from the cumulative interaction of a variety of biotic and abiotic factors and represents the animal's response to internal (physiological) and external (environmental, social) factors, relating one organism to another. In short, behavior is the how the animal "talks" to us about what it knows (Morris 2005).

Behavior is adaptable and its type, intensity, and time of occurrence within a genetically based behavioral tolerance range can be modulated, hence behavior represents an important mechanism to react and adapt to environmental changes including exposure to contaminants (Evans 1994; Dell'Omo 2002). Emlen *et al.* (1998) suggest that the remarkable ability of many organisms to adjust their physiology and behavior to day-to-day changes in the environment over a variety of scales, is primarily due to phenotypic behavioral plasticity. Behavioral plasticity, as a special case of phenotypic plasticity, is the ability of a single genotype to produce more than one alternative behavior in response to a stressor (West-Eberhard 1989).

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Three different types of behavioral plasticity can be distinguished: differences in ontogenetic development, adjustments through learning, and the innate ability to respond to a variety of stimuli (Komers 1997). In analogy to Species Sensitivity Distributions (SSDs), response curves can be established for the range of behaviors of several populations or species, performed in an environmental gradient where, for example, steep slopes express strong changes in behavior along that gradient (Komers 1997).

Behavior is a visible reaction of an organism to a stimulus on the whole-organism organization level. However, being based on biochemical reactions and exerting consequences on the population and biocoenosis levels, behavior can be regarded as highly integrative (Little 1990; Janssen *et al.* 1994; Dell'Omo 2002).

Behavior can be classified in different ways, such as: internal biochemical/physiological processes/mechanisms (neurobiological, hormonal, *etc.*); external ecological effects/consequences/purpose (*e.g.*, avoidance, mating); degree of complexity such as from simple phototaxis to foraging behavior; the distinction between individual (locomotion, foraging, learning with increasing complexity) and interactive behavior (interspecific interactions such as predator-prey, or intraspecific interaction such as aggregation, territoriality, social interaction, reproduction related behaviors such as courtship, mating, spawning and parental care, *etc.*).

BEHAVIOR IN ECOTOXICOLOGY

As outlined earlier, behavior can be seen as a visual response to a stimulus, for example, an external stimulus from the environment. Apart from natural stimuli such as changes in light and temperature, many stimuli are related to human interferences in ecosystems including release of contaminants in the environment. To study changes in behavior due to contaminant exposure is therefore an essential part of behavioral science, which can be called behavioral ecotoxicology. As contaminants are one type of environmental stressors, behavioral ecotoxicology can be seen as integral part of stress ecology (van Straalen 1997). Behavioral ecotoxicology studies how behavior is modified by environmental toxicants (Dell'Omo 2002). It is an interdisciplinary science that only gets increasing recognition if more toxicologists study behavior and more behavioral biologists study toxicology (Zala and Penn 2004) (Figure 1). Changes in animal behavior appear to be among the most sensitive indicators of environmental alterations and have been proposed as an index of sub-lethal toxicity in wildlife (Warner 1967). Behavioral changes may represent either compensatory, reversible adaptive *responses* in order to mitigate potential overt effects (*e.g.*, direct behavioral response after perception of stress) or irreversible *effects* of a toxicant on a behavioral mechanism or expression after toxicokinetic and toxicodynamic processes have started, such as AChE inhibition exerted by neurotoxins (Gerhardt 1995; Dell'Omo 2002). These two cases have already been described by physiologists for oxygen transport and aerobic metabolism (Wilson *et al.* 1994), distinguishing loading stress caused by compensatory responses to maintain homeostasis from limiting stress caused by toxic effects above the regulative capacity.

The advantages of using behavioral parameters in ecotoxicology can be summarized as follows (Gerhardt *et al.* 1994; Scherer 1992; Fossi 1998): short response times (at least for compensatory, early warning responses); sensitive (at least for toxins that

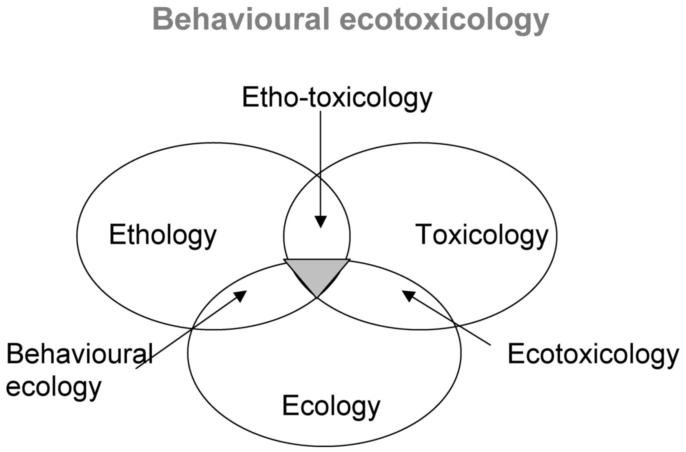

Figure 1. Interdisciplinarity of behavioral ecotoxicology (redrawn after Dell'Omo 2002).

directly affect the neuro-muscular apparatus); non-invasive, because behavior can be observed non-destructively on living organisms, for instance by inserting electrodes into the body enabling the same individual to be monitored over time by repeated measurements; adding integrative power and thus ecological relevance to laboratory toxicity tests (behavior is an excellent estimator of the "health" of a species, because behavioral abnormalities affect intra- and inter-specific interactions); allowing for time-dependent data analysis such as TTR (Time-To-Response) in analogy to TTD (Time-To-Death), which is often more sensitive than the fixed endpoint analysis, for instance an EC_{50}. The study of locomotory behavior is attractive both because it belongs to the basic behavioral repertoire in all animal taxa, and because different degrees of complexity within locomotion (*e.g.*, spontaneous, forced and reactive locomotion, locomotion in function of feeding and social interaction behavior; Dell'Omo 2002) can easily be quantified and automated.

BEHAVIOR AND OTHER BIOMARKERS

Compared to survival as an endpoint, behavioral parameters have been proven to be often between 10–100 times more sensitive. Most of these studies refer to fish and consider swimming, ventilation, and foraging behavior (Beitinger 1990; Beitinger and McCauley 1990; Dell'Omo 2002) (Figure 2).

In some cases (*e.g.*, fish) behavioral endpoints can be as sensitive as biochemical and physiological biomarkers (Passino-Reader *et al.* 1995; Triebskorn *et al.* 1997). However, direct comparisons are difficult, as few studies have simultaneously measured an array of different biomarkers and linked their responses to different biological organization levels. In some cases, behavior has been linked to other biomarkers such as neurotransmitters, plasma enzymes, mixed function oxidases, hormones, energy metabolism, and so on (Dell'Omo 2002).

Comparing behavioral with morphological endpoints in the insect larvae *Chironomus riparius* revealed that deformed larvae showed a different behavior than normal larvae, when exposed to polluted water; however, deformed larvae that were

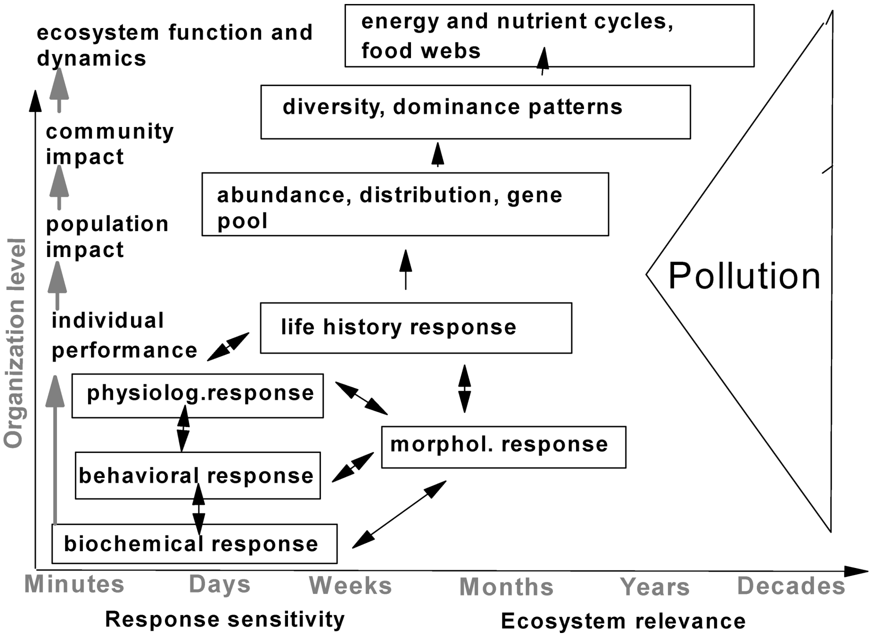

Figure 2. Biological responses at different biological organization levels plotted versus time scale for response times.

genetically adapted to historic pollution did not show any differences in behavior compared to normal larvae (Gerhardt and Janssens de Bisthoven 1995).

The "Multiple Response Concept" (Depledge 1994), recommending studying and linking biomarkers at different suborganismal and organismal levels, is still relevant and should be applied in ecotoxicological research wherever possible. We need more of these studies linking different types of biomarkers from different biological organization levels in order to "complete the cycle of evidence" in a "weight of evidence" based approach in risk assessment (Dell'Omo 2002).

Ecological relevance of biomarkers *sensu latu* is generally considered to increase with the degree of biological complexity (Adams 1990); however, pollution specificity decreases in the same direction (Peakall 1994). Nevertheless, some links between pollution type and behavioral effects have been established as for AChE inhibitors (especially effects of organophosphates on fish, beetles, birds, and mammals) and endocrine disruptors (Dell'Omo 2002). Behavioral endpoints are also used to detect potential neurotoxic effects of chemicals in humans and fish.

Drummond and Russom (1990) categorized over 300 organic chemicals from 49 chemical classes according to three acute behavioral toxicity syndromes in fish: (1) hypoactivity (decreased locomotion, loss of startle response) often linked to narcotic action; (2) hyperactivity (accelerated locomotion, overreaction to stimuli, increased ventilation) often linked to disruption in metabolic function; and (3) physical abnormality (convulsions, bone deformities) often linked to damage to the nervous system. Multiple syndromes were observed for most of the chemical classes. Metals can have several sites and modes of toxic action including interference with biochemical processes, damage of nervous or epithelial tissues (*e.g.*, gills, olfactory epithelium), hence leading to many behavioral alterations (Dell'Omo 2002; Gerhardt 1993).

RECENT DEVELOPMENTS

Technology to Automate Data Recording

Before the technical revolution, behavioral studies were performed by visual observation and manual data analysis. This was very time-consuming and sometimes even impossible (*e.g.*, during the night). Time-lapse photography and video-filming (2-D, 3-D) have been developed during the last decades as a basis for image analysis software and are mostly used in terrestrial behavioral research on small mammals (for instance, mazes) and humans (for instance, the recent development of facial expression analysis). Infra-red light beams have also been used to record locomotion of aquatic animals. Even though the development of non-optical methods has been less fast than that of image recording and analysis, non-optical methods have several advantages in certain situations, such as: recording in turbid water, soil, or sediment; able to operate without an additional light source, which might alter the normal behavior of the test species; often more robust and less expensive for applications *in situ*. Examples of non-optical methods are ultrasound beams, magnetic inductance, tracking sensors, telemetry, neural microstimulation methods combined with action potential recordings, harmonic radar tracking systems, PIT-tags (passive integrated transponder), and impedance conversion technology (Gerhardt *et al.* 1994; Gerhardt 1999; Ballintijn *et al.* 2005). Some of these technologies have been the basis for developing biomonitoring systems to continuously monitor short-term changes in water quality, based on behavioral stress responses of indicator species. One example of a non-optical online biomonitor is the Multispecies Freshwater Biomonitor^{TM}, based on quadropole impedance technology (Gerhardt *et al.* 1994, 2006a). Behavioral endpoints are used in evaluating pulse pollution, as applied in the so-called BEWS (Biological Early Warning Systems) or on-line biomonitors. Such instruments are being continuously developed and installed along large rivers in Europe, often also at country borders in order to detect pollution, pursue polluters and warn downstream monitoring stations in due time (Gerhardt 1999). Automated behavioral tests such as the earthworm avoidance test (Yeardley *et al.* 1996) and the altered locomotory behavior of woodlice (Sorensen *et al.* 1997) are valuable to show when organisms are exposed to contaminants at biologically significant levels (Dell'Omo 2002).

New Methods for Treating and Analysing Behavioral Data

Statistical analysis and inference hypothesis testing is often difficult as behavioral data often do not meet the necessary assumptions. For instance, bimodality and divergence in behavior are best analysed by models based on catastrophe theory (Koene 2005). Often behavioral data contain several problems, such as non-independent observations (apply autocorrelation coefficient, MANOVA), non-normal distribution and heterogeneity of variances (apply non-parametric methods), multiple correlated response variables (data analysis by PCA, time series models) and unbalanced groups and missing values due to mortality (apply General Linear Model) (Dell'Omo 2002).

New approaches include time series analysis and modelling, detection of anomalies due to pollution pulses by jump detectors (Hinkley detector), or neuronal networks and fuzzy logic. Methods such as breakpoint analysis, proportional fitting and sequential pattern analysis have been developed for data matrices and are commercially available (Ballintijn *et al.* 2005). Often behavioral data have to be pre-treated by smoothing procedures and noise reduction methods, such as diverse software filters. Often the amount of data is so large that data reduction for instance by PCA, factor analysis or summarizing of data by mean values is needed.

An Example: Applications of the Recent Development in the MFB

The MFB (Multispecies Freshwater Biomonitor^{TM}) is based on a non-optical recording principle, the so-called quadropole impedance conversion technology, where a pair of steel electrodes generates a high frequency electrical field over a test chamber filled with medium and an organism, and a second pair of electrodes records the changes in this field provoked by movements of the organism (Gerhardt *et al.* 1994) (Figure 3). The MFB applies modern time-series analysis as well as jump detection to the data, in order to detect behavioral changes, and a recently developed neuronal network method is being compared with those existing methods.

As a non-optical method the MFB does not need additional light sources, which might alter the normal behavior of animals, such as diurnal migration patterns, or exert additional stress, such as for negatively phototactic benthic species. The MFB has been used to study artificially induced phototaxis in the chironomid *Procladius choreus* (Gerhardt and Janssens de Bisthoven 2000) as well as phototaxis in combination with diurnal vertical migration of *Daphnia magna* (Gerhardt *et al.* 2006b), and complex behavioral rhythms with high ecological significance such as predator avoidance. Artificial phototaxis by induced light in intervals of 2 hours was shown by the waterfleas, even during the night periods, hence both behaviors, phototaxis and diurnal vertical migration, overlapped.

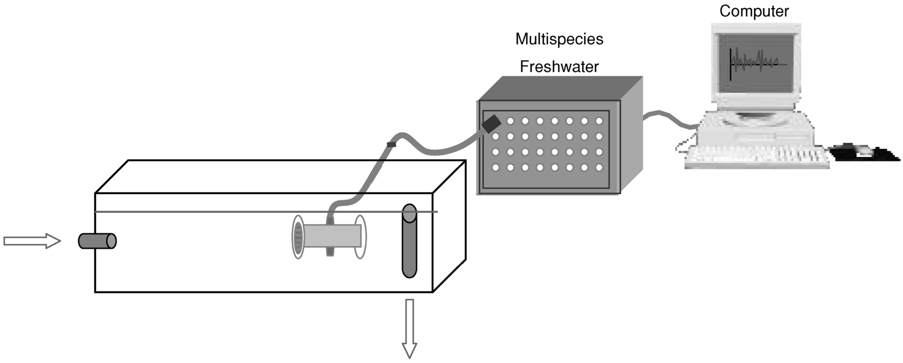

Figure 3. The Multispecies Freshwater Biomonitor^{TM} (LimCo International, Germany) consisting of flow-through tank, test chambers with individual animals connected to the MFB-impedance recorder instrument, and the computer for data analysis.

The MFB has been applied in different media, water, soil, and sediment, hence it is able to record the behavior of animals in their natural substrate, which is not possible by optical methods. Behavioral changes have been recorded in several aquatic invertebrates and vertebrates exposed to metals, acidity and/or complex water, such as seven freshwater crustacean species (Gerhardt 2001), as well as the marine crustacean *Corophium volutator*. Moreover, four small freshwater fish species have been used in the MFB. Sediment toxicity tests are being developed for *Chironomus riparius,* as well as the freshwater oligochaetes *Tubifex tubifex* and *Lumbriculus variegatus.* Soil species have recently been used to test narcotic chemicals (*Porcelio scaber*) and effects of salinisation (*Lumbricus terrestris*) (Gerhardt and Filser unpublished data). The use of natural substrates is recommended for sediment and soil toxicity tests because: the behavior of the organisms is more normal than in media that they normally do not live in; bioavailability of toxicants is closer to what happens in the natural environment instead of, for example, using extracts or pore water; and the animals are less stressed by the artificial test surroundings, which might lead to overestimation of the potential toxicity of the substance being tested. All our studies showed that behavioral responses of invertebrates and fish are sensitive to toxicants with response times between 30 min to several hours depending on site and mode of action of the toxicants when exposed alone, compared to longer response times when animals were exposed to complex waste water containing several interacting chemicals.

Especially for fish and Crustacea a typical sequence of behaviors can be observed, as described in the empirical Stepwise Stress Model (SSM) (Gerhardt 1999; Gerhardt *et al.* 2005) (Figure 4). Plotting increased toxicant contamination on the x-axis, we can define ranges of toxicant concentrations where no behavioral response occurs (resistance), where a compensatory (regulative) response occurs (regulation) and where a serious irreversible effect occurs (irreversible toxic effect), leading to persistent damage. Responses of two types of behavior (locomotion, ventilation) are plotted on the y-axis at different levels of overall response intensity. Ventilation is

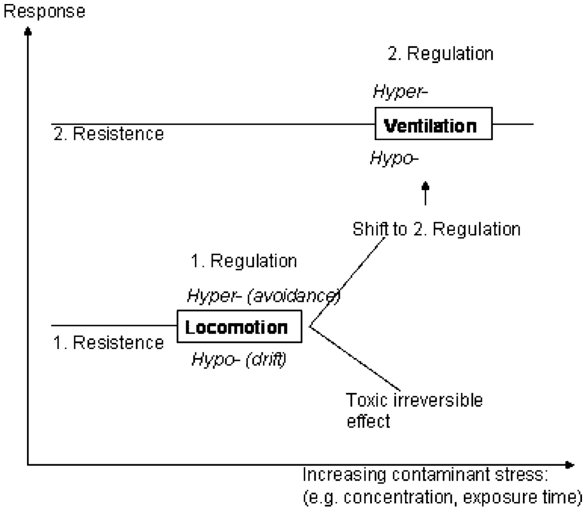

Figure 4. Stepwise Stress Model.

plotted at a higher response intensity than locomotion, as we found in our studies that behavioral responses in ventilation are always performed after other behavioral responses have taken place, meaning that ventilation responses need a higher threshold of both response intensity and contaminant concentration. Within both response types (locomotion, ventilation) we can see the inherent ranges for resistance, regulation and irreversible effects. Within the range of regulation, there are always two options for an animal to respond, either with increasing behavioral response (*e.g.*, increasing locomotion, increasing ventilation) or with decreasing behavioral response. Whereas increasing locomotion is defined here as avoidance sensu strictu (*i.e.*, locomotory escape behavior), decreasing locomotion (often resulting in passive drift) rather represents a narcotic response. According to this model, the first behavioral response after contact with a toxicant is an avoidance reaction (*e.g.*, increased locomotory activity in order to escape from the site), followed by a second behavioral response consisting of increased ventilation with the gills, to get rid of toxins from the respiratory surfaces (Figure 4). Increased ventilation can be recorded as increased time spent on ventilation, but often also as increased ventilation frequency, that is, the organisms hyperventilate. Such behavioral early warning responses have also been applied in *in situ* online biomonitoring with the MFB, for example, monitoring of water from the River Rhine with *Gammarus* sp. and *Dinocras cephalotes* (Gerhardt unpublished) and water intake for drinking water production at the River Rhine with *Daphnia magna* and *Gammarus pulex* (Gerhardt *et al.* 2003) in order to detect irregularities in water quality as well as toxic pulses. Moreover, the SSM supports the concept of behavioral plasticity because it is anticipated that species having a sequential stepwise response pattern of different stress behaviors are more tolerant against environmental stress than species with only one stress response type.

The SSM as described here is an empirical model for early warning responses within the species-specific inherent regulative range of a behavioral trait, that is, direct behavioral responses after perception of the stressor within a few hours of exposure, and not for irreversible behavioral effects after toxicokinetic and toxicodynamic processes. A mathematical description of the Stepwise Stress Model has been proposed (Gerhardt *et al.* 2005).

An example of species showing only one behavioral stress response is the freshwater oligochaete *Tubifex tubifex*, which was exposed during acute exposure (24 h) to a range of concentration levels of toxicants from different chemical classes, such as metals, pesticides, and pharmaceuticals, but the only behavioral response recorded was a decrease in locomotion at almost lethal concentration levels (Gerhardt 2006). *T. tubifex* hence shows only one stress response, at high exposure levels. On the other hand, species following the SSM have include the crustaceans *Gammarus pulex* and *Macrobrachium nipponense*, the mayfly *Adenophlebia auriculata,* and the fish *Oryzias latipes* and *Gambusia holbrooki* (Gerhardt *et al.* 2005).

An example of how to integrate a behavioral biotest in a monitoring program is provided by Gerhardt *et al.* (2004) in a multimetric study of an acid mine drainage effluent in Portugal, where bioassessment was established by different methods based on ecosystem structure and function, chemical assessment based on analysis of several metals and salts, and toxicity assessment was based on laboratory and *in situ* tests in the MFB with standard (*Daphnia magna, Chironomus* sp., *Lemna gibba)* and locally resident species (*Gambusia holbrooki, Atyaephyra desmaresti, Choroterpes picteti*).

As a result, a new multimetric index was developed, which better distinguished the sites than bioassessment methods alone. This shows how behavioral toxicity tests can easily be included in water quality bioassessment and online biomonitoring of point pollution sources, hence representing valuable methods to be established in the European Water Framework Directive.

SUMMARY: PROS AND CONS OF BEHAVIORAL ECOTOXICOLOGY

Behavioral tests have a high potential to be applied in ecotoxicological research as well as in biomonitoring, in addition to other biological and chemical methods in a sound triad-based multimetric approach. They offer ecologically relevant, sensitive, fast and non-destructive tests, which can be quantified and automated in order to achieve time- and cost-effective test systems.

Behavioral ecotoxicology is expanding due to emerging technologies for automated recording and data analysis. Implementation of behavioral tests in a regulatory framework has started, such as the adoption of certain behavioral tests (*e.g.*, lethargy, tremors, fish), for US National Resource Damage Assessments (NRDAs) as well as the implementation of on-line biomonitors along large rivers for monitoring water quality (Gerhardt 1999); hence, implementation in the European Waterframework Directive and European Soil Strategies is recommended.

However, some problems have still to be solved, mainly concerning field validation, linking behavioral to other biological and ecological responses as well as standardization.

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