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Optimising a clearance index based on neutral red as an indicator of physiological stress for bivalves

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ABSTRACT

Neutral red is a weakly cationic dye that is soluble in water, has a low toxicity for almost all classes of organisms, and has been used as a histological and vital stain since the early twentieth century. Estimating the volume of water cleared of indicator material by suspension feeding bivalves (clearance or filtration rate) was one of the earliest applications of neutral red; however, less than thirty studies have applied this methodology since it was first described in 1954. The feeding/clearance rate is used as a sensitive indicator of physiological stress and is therefore an early-warning tool that is suitable for monitoring the ecological status of water bodies. The aim of our study was to optimise a clearance index based on neutral red solution by addressing the effect of i) the acidifying pH; ii) the holding temperature before spectrophotometric reading; and the time iii) before and iv) after the acidification of solutions of neutral red used to carry out clearance assays. Furthermore, as a case study we fine-tuned the clearance assay for the edible estuarine bivalve, *Cerastoderma edule*. The results showed that there were no statistical differences as regards the absorbance of neutral red solutions holding at 4 or 20 °C or a solution acidified between the ranges of pH 4–5. However, the absorbance significantly decreased as the pH increased to pH 6. The time before acidification had no significant effect on absorbance. Once the neutral red solution is acidified, the absorbance decreases over time, signifying that the absorbance should be read in the first 24 h. The concentration of neutral red used in the experiences should be sufficient to allow final concentrations of over 0.5 mg/L after the clearance period, since we observed that the sensitivity of this protocol decreased at low concentrations. In the case of *C. edule*, the optimum clearance conditions per individual were found to be 100 ml of 4 mg/L of neutral red dye during a 30 min period in dark conditions. A bioassay using a clearance index of *C. edule* based on this simple colorimetric technique would appear to be a potential tool for implementation in environmental monitoring programmes for water quality assessment in accordance with European directives. We trust that the new harmonised protocol will become a widely used and cost-effective means to monitor the clearance index as an indicator of physiological stress for bivalves.

1. Introduction

Neutral red or toluylene red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a tricyclic aromatic amino-azin derivate of chromogenphenazine (Conn, 1961). Neutral red is a weakly cationic dye that is soluble in water, which has a low toxicity for almost all classes of organisms (Barbosa and Peters, 1971; Kastury et al., 2015), and has therefore been used as a histological and vital stain since the early twentieth century (Koehring, 1930). Besides its wide uses in histology, neutral red also has multiple applications in very different disciplines; see Barbosa and Peters (1971), and Table 1 for a list of the most common applications.

One of the earliest applications of neutral red was that of estimating the amount of water filtered by bivalves for comparative purposes (Cole and Hepper, 1954). The measurement of the rate at which an organism clears a suspension of indicator material is one of the indirect methodologies most frequently used to study the feeding rate in suspension feeding bivalves (Riisgard, 2001). The amount of water cleared of particles per unit of time is defined as the clearance rate, which for particles that are retained by the ani-

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Table 1
Applications of neutral red dye solutions.

Application	Reference
Algae differentiation	Elliott and Tang (2009)
Animal distribution and population measurements	Lawler and Fitz-Earle (1968)
Biomarker of cell damage	Borenfreund and Puerner (1985)
Catalyser of reductive dechlorination	Yee et al. (2010)
Confocal probe in botanic	Dubrovsky et al. (2006)
Detector/differentiator bacteria (reduction)	Hunter (1901)
Electron mediator for electricity generation	Park and Zeikus (2000)
Enhanced biogas production	Beckmann et al. (2014)
Filtering rate indicator	Cole and Hepper (1954)
Intracellular pH indicator	LaManna and McCracken (1984)
Lipid fluorochrome	Kirk (1970)
Marine plankton differentiation	Crippen and Perrier (1974)
Optical pH sensors	Goicoechea et al. (2008)
Textile dye	Guiza et al. (2004)

mal's filter with 100% efficiency is equivalent to the filtering rate, or filtration rate (Riisgard and Larsen, 2000). Cole and Hepper (1954) aimed to replace laborious techniques previously developed for the comparative study of feeding rates of bivalves, which appeared to have various drawbacks (namely, the need for many workers, complicated apparatus, and/or the careful preparation of the material; Cole and Hepper, 1954). The methodology described by these authors was related to the properties of neutral red as an intra-vital stain, and was therefore based on the absorption and retention of dye by the gills of bivalves. In this respect, Cole and Hepper (1954) stated that the amount of neutral red removed from the water would appear to depend directly upon the volume of water pumped through the gills. The concentration of neutral red can easily be measured by acidifying the solution with a few drops of HCl, owing to the properties of neutral red as a pH indicator (e.g., LaManna and McCracken, 1984). Neutral red solution becomes pink in acidic conditions making it suitable for colorimetric comparisons. Although the study of feeding rate is widely used in physiological, ecological and/or ecotoxicological studies focused on suspension feeding bivalves, we found less than thirty studies in which the technique based on neutral red solution had been applied (see Table 2).

Physiological responses to stress, such as feeding (clearance/filtering rate), are considered suitable parameters to be implemented when monitoring water quality schemes in accordance with European directives because they provide valuable information on the organismís condition in the systems (Baird et al., 2007). Feeding inhibition can be used as a sensitive indicator of exposure to pollutants, since it detects the effects of stress at lower pollutant levels than when using mortality rates alone (Abel, 1976; Martinez-Haro et al., 2014; McLoughlin et al., 2000; Melvin and Wilson, 2013). This may facilitate early warning, indicating that toxic conditions are occurring before population dynamics become affected (Maltby et al., 2002; McWilliam and Baird, 2002).

Numerous methodologies based on direct and indirect methodologies have been described to study the feeding activity in suspension feeding bivalves (e.g., Coughlan, 1969; for an extensive review, see Riisgard, 2001). Indirect methodologies based on clearance methods have been carried out using very different indicator material, e.g., algae, graphite, blood protein or dye solutions, of which those using algae solutions are the most frequent (e.g., Foster-Smith, 1975 Jørgensen, 1960). Algae growths are highly dependent on specific environmental conditions, such as light, water temperature, nutrient concentration, salinity, and/or pH (Singh et al., 2015). Moreover, the use of algae solutions involves algae cultivation and maintenance under laboratory conditions, which is laborious, time consuming and requires specific laboratory infrastructures/facilities and qualified staff (Singh et al., 2015). Other interesting alternative techniques are cost-effective method-

Table 2
Works in which the technique based on neutral red solution has been applied in suspension feeding bivalves since its description by Cole and Hepper (1954).

Year	Reference	Species	Factors tested
1954	Cole and Hepper, 1954	*Mytilus edulis*	T, S, parasitation
1956	Nagabhushanam, 1956	*Martesia striata*	S, light
1960	Matthiessen, 1960	*Mya arenaria*	S
1963	Durve, 1963	*Meretrix casta*	Size, S
1967	Salánki and Lukacsovics, 1967	*Anodonta cygnea*	Oxygen
1973	Ansell and Sivadas, 1973	*Donax vittatus*	T
1973	Ward and Aiello, 1973	*Mytilus edulis*	SM
1975	Badman, 1975	*Actinomalas carinata, Elliptio dilatatus, Pleurobema coccineum*	Oxygen
1975	Mane, 1975	*Katelysia optima*	T, S, pH, tidal cycle, SM, light, H^+, size, parasitation, siphon tip cut
1976	Abel, 1976	*Mytilus edulis*	Cu, Zn, Hg, cyanide, thiocyanate, suphide
1976	Alagarswami and Victor, 1976	*Pinctada fucata*	S
1978	Bedford et al., 1978	*Crassostrea glomerata*	
1979	Pregenter, 1979	*Mytilus edulis*	Presence of the crab *Pinnotheres hickmani*
1981	Watling, 1981	*C. gigas, C. margaritacea, Perna perna, Choromytilus meridionalis*	Cu, Zn, Cd, Pb
1982	Watling and Watling, 1982	*Perna perna*	Ni, Co, Cr, Ag, Mn, As, Hg, Se
1989	Patel and Eapen, 1989	*Anadara granosa*	Naphthalene
1990	Micallef and Tyler, 1990	*Mytilus edulis*	Hg, Se
1990	Krishnakumar et al., 1990	*Perna viridis*	Cu, Hg
2008	Kang et al., 2008	*Scapharca broughtonii*	Size, T, S
2009	Faria et al., 2009	*Dreissena polymorpha*	PCBs (Aroclor 1260), metals (Cd, Hg)
2012	Palais et al., 2012	*Dreissena polymorpha*	Field samples
2014	Parolini et al., 2014	*Dreissena polymorpha*	MDMA (3,4-methylenedioxymethamphetamine)
2016	Magni et al., 2016	*Dreissena polymorpha*	Morphine

T = temperature, S = salinity, SM = suspended matter.