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Pim de Voogt *Editor*

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Reviews of Environmental Contamination and Toxicology

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Volume 240

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Foreword

International concern in scientific, industrial, and governmental communities over traces of xenobiotics in foods and in both abiotic and biotic environments has justified the present triumvirate of specialized publications in this field: comprehensive reviews, rapidly published research papers and progress reports, and archival documentations These three international publications are integrated and scheduled to provide the coherency essential for nonduplicative and current progress in a field as dynamic and complex as environmental contamination and toxicology. This series is reserved exclusively for the diversified literature on "toxic" chemicals in our food, our feeds, our homes, recreational and working surroundings, our domestic animals, our wildlife, and ourselves. Tremendous efforts worldwide have been mobilized to evaluate the nature, presence, magnitude, fate, and toxicology of the chemicals loosed upon the Earth. Among the sequelae of this broad new emphasis is an undeniable need for an articulated set of authoritative publications, where one can find the latest important world literature produced by these emerging areas of science together with documentation of pertinent ancillary legislation.

Research directors and legislative or administrative advisers do not have the time to scan the escalating number of technical publications that may contain articles important to current responsibility. Rather, these individuals need the background provided by detailed reviews and the assurance that the latest information is made available to them, all with minimal literature searching. Similarly, the scientist assigned or attracted to a new problem is required to glean all literature pertinent to the task, to publish new developments or important new experimental details quickly, to inform others of findings that might alter their own efforts, and eventually to publish all his/her supporting data and conclusions for archival purposes.

In the fields of environmental contamination and toxicology, the sum of these concerns and responsibilities is decisively addressed by the uniform, encompassing, and timely publication format of the Springer triumvirate:

Reviews of Environmental Contamination and Toxicology [Vol. 1 through 97 (1962–1986) as Residue Reviews] for detailed review articles concerned with any aspects of chemical contaminants, including pesticides, in the total environment with toxicological considerations and consequences.

Bulletin of Environmental Contamination and Toxicology (Vol. 1 in 1966) for rapid publication of short reports of significant advances and discoveries in the fields of air, soil, water, and food contamination and pollution as well as methodology and other disciplines concerned with the introduction, presence, and effects of toxicants in the total environment.

Archives of Environmental Contamination and Toxicology (Vol. 1 in 1973) for important complete articles emphasizing and describing original experimental or theoretical research work pertaining to the scientific aspects of chemical contaminants in the environment.

The individual editors of these three publications comprise the joint Coordinating Board of Editors with referral within the board of manuscripts submitted to one publication but deemed by major emphasis or length more suitable for one of the others.

Coordinating Board of Editors

Preface

The role of *Reviews* is to publish detailed scientific review articles on all aspects of environmental contamination and associated (eco)toxicological consequences. Such articles facilitate the often complex task of accessing and interpreting cogent scientific data within the confines of one or more closely related research fields.

In the 50+ years since *Reviews of Environmental Contamination and Toxicology* (formerly *Residue Reviews)* was first published, the number, scope, and complexity of environmental pollution incidents have grown unabated. During this entire period, the emphasis has been on publishing articles that address the presence and toxicity of environmental contaminants. New research is published each year on a myriad of environmental pollution issues facing people worldwide. This fact, and the routine discovery and reporting of emerging contaminants and new environmental contamination cases, creates an increasingly important function for *Reviews.* The staggering volume of scientific literature demands remedy by which data can be synthesized and made available to readers in an abridged form. *Reviews* addresses this need and provides detailed reviews worldwide to key scientists and science or policy administrators, whether employed by government, universities, nongovernmental organizations, or the private sector.

There is a panoply of environmental issues and concerns on which many scientists have focused their research in past years. The scope of this list is quite broad, encompassing environmental events globally that affect marine and terrestrial ecosystems; biotic and abiotic environments; impacts on plants, humans, and wildlife; and pollutants, both chemical and radioactive; as well as the ravages of environmental disease in virtually all environmental media (soil, water, air). New or enhanced safety and environmental concerns have emerged in the last decade to be added to incidents covered by the media, studied by scientists, and addressed by governmental and private institutions. Among these are events so striking that they are creating a paradigm shift. Two in particular are at the center of ever increasing media as well as scientific attention: bioterrorism and global warming. Unfortunately, these very worrisome issues are now superimposed on the already extensive list of ongoing environmental challenges.

The ultimate role of publishing scientific environmental research is to enhance understanding of the environment in ways that allow the public to be better informed or, in other words, to enable the public to have access to sufficient information. Because the public gets most of its information on science and technology from internet, TV news, and reports, the role for scientists as interpreters and brokers of scientific information to the public will grow rather than diminish. Environmentalism is an important global political force, resulting in the emergence of multinational consortia to control pollution and the evolution of the environmental ethic. Will the new politics of the twenty-first century involve a consortium of technologists and environmentalists, or a progressive confrontation? These matters are of genuine concern to governmental agencies and legislative bodies around the world.

For those who make the decisions about how our planet is managed, there is an ongoing need for continual surveillance and intelligent controls to avoid endangering the environment, public health, and wildlife. Ensuring safety-in-use of the many chemicals involved in our highly industrialized culture is a dynamic challenge, because the old, established materials are continually being displaced by newly developed molecules more acceptable to federal and state regulatory agencies, public health officials, and environmentalists. New legislation that will deal in an appropriate manner with this challenge is currently in the making or has been implemented recently, such as the REACH legislation in Europe. These regulations demand scientifically sound and documented dossiers on new chemicals.

Reviews publishes synoptic articles designed to treat the presence, fate, and, if possible, the safety of xenobiotics in any segment of the environment. These reviews can be either general or specific, but properly lie in the domains of analytical chemistry and its methodology, biochemistry, human and animal medicine, legislation, pharmacology, physiology, (eco)toxicology, and regulation. Certain affairs in food technology concerned specifically with pesticide and other food-additive problems may also be appropriate.

Because manuscripts are published in the order in which they are received in final form, it may seem that some important aspects have been neglected at times. However, these apparent omissions are recognized, and pertinent manuscripts are likely in preparation or planned. The field is so very large and the interests in it are so varied that the editor and the editorial board earnestly solicit authors and suggestions of underrepresented topics to make this international book series yet more useful and worthwhile.

Justification for the preparation of any review for this book series is that it deals with some aspect of the many real problems arising from the presence of anthropogenic chemicals in our surroundings. Thus, manuscripts may encompass case studies from any country. Additionally, chemical contamination in any manner of air, water, soil, or plant or animal life is within these objectives and their scope.

Manuscripts are often contributed by invitation. However, nominations for new topics or topics in areas that are rapidly advancing are welcome. Preliminary communication with the Editor-in-Chief is recommended before volunteered review manuscripts are submitted. *Reviews* is registered in WebofScience™.

Inclusion in the Science Citation Index serves to encourage scientists in academia to contribute to the series. The impact factor in recent years has increased from 2.5 in 2009 to almost 4 in 2013. The Editor-in-Chief and the Editorial Board strive for a further increase of the journal impact factor by actively inviting authors to submit manuscripts.

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A Review on the Abundance, Distribution and Eco-Biological Risks of PAHs in the Key Environmental Matrices of South Asia

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1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of ubiquitous persistent organic pollutants (more than 100 congeners) comprised of two or more benzene rings arranged in various configurations. This class of pollutant has gained much attention of researchers worldwide because of their recognized carcinogenic, mutagenic and immuno-toxic properties (Din et al. 2013; Callén et al. 2011; Jang et al. 2013). Human/biota is exposed to PAHs in both residential and occupational settings, rural/urban area and outdoor and indoor environment (Ravindera et al. 2007; Kim et al. 2013; Kamal et al. 2015). Different international environmental agencies (i.e., ATSDR, US EPA) have identified a list of priority pollutants, based on their abundance in the exposure sites, and known health effects (Kamal et al. 2015). PAHs have also been recently incorporated into the list of protocol to the convention on long range trans-boundary air pollution protocol on persistent organic pollutants (Zhang and Tao 2009).

Occurrence of PAHs in various environmental compartments is attributed to both natural (volcanoes and forest fires) as well as to anthropogenic sources (refining and spillage of petroleum and its related products, exhaust of motor vehicles, coal and coke combustion etc.) (Ravindera et al. 2007; Zhang and Tao 2009). The ubiquity of these chemicals in the environment is facilitated by several mechanisms such as evaporation, dry and wet deposition, runoff and precipitation and allowing them to distribute in several environmental matrices i.e., soil, sediment, air, water, biota and human adipose tissues (Baklanov et al. 2007; Kim et al. 2013; Kamal et al. 2015). A conceptual model of PAHs compounds across various environmental compartments has been presented in Fig. 1.

Over the last few decades, a rapid industrialization and urbanization was observed in the South Asian countries, especially in India, Pakistan, Nepal, Sri Lanka and Bangladesh. Along with these factors, there have been a continuous increase in the population growth, a gradual increase in the number of vehicles on roads and associated emission releases of PAHs in this region (Masih and Taneja 2006). In addition, the exhaust emissions from industrial sector and biomass burning have also contributed to atmospheric emissions of PAHs. According to a report of World Health Organization (WHO), wood, dung-cakes and agricultural residues are commonly used for cooking in developing countries and accounting for more than 75 % of energy demands in India (WHO and IPCS 1998). Among the fossil fuels, diesel/gasoline are further sources of PAHs in particular, in the urban

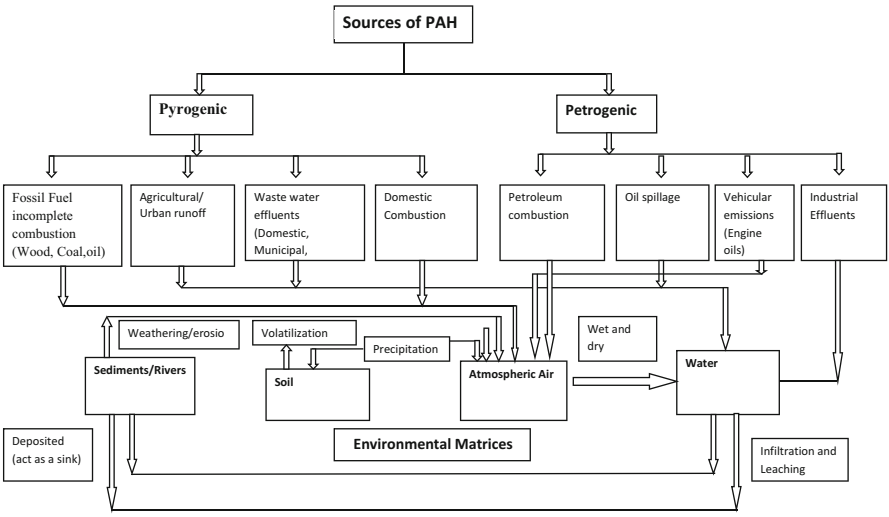

Fig. 1 Conceptual framework of PAHs pathways across various environmental compartments

areas (Sharma et al. 2008). Limited literature reports on the rate of emissions, diversity of sources and distribution of PAHs in the South Asian region have resulted in an uncertainty and ambiguous situation compared with those reported in developed countries. PAHs in the atmosphere of South Asia vary both geographically and seasonally. Variability on atmospheric concentrations is probably associated with the seasonal variations, in which regional scale winds (the monsoon) play a key role in the transport of contaminants. Regional scale winds circulate due to unequal heating of air over the continents and oceans in summer and winter seasons (Ali et al. 2014). It's like a cyclic phenomenon which reverses on the seasonal basis along with the patterns of air flow. In general, the continents are more heat up in the summer season than the oceans and vice-versa. This temperature gradient creates high and low pressure zones in the atmosphere, stimulates the air circulation and it is responsible for the transportation of the organic contaminants. In winter season the continent cools rapidly forming a large area of high pressure over north central Asia and a smaller area over India.

Air movements transport small contaminated particles which increase the levels of PAHs concentrations (Genualdi et al. 2009). According to Wania and Mackay (1993), long range atmospheric transport (LRAT) is the main global transport pathway for PAHs; in fact, in warm regions, these compounds tend to evaporate into the atmosphere and to be transported long distances before ending up on cold surfaces in the polar regions. Moreover, lower mixing layer and less intensive atmospheric reactions govern the change in the behavior of atmospheric concentrations.

Friedman et al. (2013) investigated the influence of climate change on the atmospheric transport of the PAHs to the arctic region. For this purpose, they used GEOS-Chem meteorology coupled model and determined a General

Circulation Model Simulations (GCM) (2000–2050) in relation with PAHs emissions from northern hemisphere (mid Latitudes) to the Arctic. Authors revealed that in addition to the LRAT property, the local anthropogenic activities in Arctic region, such as oil spills, oil/gas shipping, domestic combustion, were also contributing to PAHs levels. Vapor pressure is among key properties of PAHs, which influences the soil-air and air-water exchanges as well as their vapor-particle partitioning. This property has also a fundamental role in the LRAT of PAHs and their phase transfer into soil organic matter. Scavenging of atmospheric PAHs from snow/ice is a known phenomenon for atmospheric removal (Gabrieli et al. 2010). Ice melting increases the possibility of air-water exchange, with consequent incorporation of particulate PAHs into ocean. It was evidenced from the changing concentrations in phytoplankton and oceanic organic carbon (Wania et al. 1998, 1999). Inomata et al. (2012), estimated the particulate PAHs levels in the northeastern Asia using an aerosol chemical transport model and revealed an abetment in the trans-boundary translocation of pollutants during summer season. During winter season, an increase in concentration was probably due to the high temperature inversions which take place in the cold arctic surfaces (Halsall 2004). PAHs can travel long distances through air, water and soil particles, and eventually accumulate in cold high altitude regions, which act as a cold condenser for this kind of volatile compounds. The Himalayas, the highest mountain range in the world, cover the belt of River Indus in Pakistan, touchdown east wards towards India, extend along the Southern Tibet, cover the Nepal and end at Bhutan. The Himalayas, with their thousands of glaciers, harbor water reservoirs for the great rivers of China, India and other Asian countries, is also called the Third Pole, and similarly to the Arctic region it behaves as condenser for PAHs (Kang et al. 2009). Yuan et al. (2015), recently measured PAHs contents in soils of Central Tibetan Plateau (CTP), China revealed that PAHs levels were decreased with increasing distances from the source. Meanwhile the proportion of light fraction, PAHs were increased with increasing distances. Similar trends was observed from a decade of monitoring in the Canadian Arctic, confirmed that light fraction of PAHs might be responsible for atmospheric long range transport (Hung et al. 2005). However, continuous monitoring of organic pollutants may lead to a better understanding of local and global transport patterns (Gabrieli et al. 2010).

Unlike several developed nations, the permissible limits of PAHs exposure are scarcely available in many countries of the South Asia, which can be ascribed to the unavailability of the technical and laboratory facilities in this region. The current review encompasses the available information on the levels, distribution, and possible sources and associated eco-toxicological risks of PAHs which were detected in different environmental media of South Asian countries. The review provides currently available baseline data on PAHs of this region, and therefore could be helpful in identifying the existing research needs and data gaps.

2 Abundance of PAHs in SAR

2.1 Atmospheric Levels

Occurrence of PAHs in the urban air has caused particular concern because of the continuous nature of exposure and the size of population at risk. Human can be affected by direct inhalation of air pollutants, tobacco smoke, or through the ingestion of contaminated/processed food/water, and also through dermal contact (Kim et al. 2013).

In general, the atmospheric PAHs come through direct emissions from pyrogenic and petrogenic sources and/or volatilization from the contaminated water bodies and soil surfaces (Zhang et al. 2009). In SAR, previous studies have reported atmospheric distribution of PAHs in developing South Asian countries, including India, Nepal, Pakistan and Sri Lanka (Karunaratne and Wijayarathna 2013; Ravindra et al. 2007; Kishida et al. 2009) (Table 1). In some countries, such as India, the level of PAHs is strongly influenced by the meteorological conditions, such as low atmospheric temperature which reduces the atmospheric dispersion of pollutants. In India, this kind of dispersion is facilitated by the formation of a stable atmospheric layer that leads to high pollutant load during winter season in Agra (Fig. 2). On contrary, a high temperature during summer season usually results in the increased dispersion rate which reduces the concentrations of such pollutants. However, other meteorological parameters includes wind speed, rainfall, humidity might influence the concentration pattern (Likhani 2012). A similar observation in Delhi was reported by Sharma et al. (2007), which showed that the average seasonal concentration of total PAHs remained maximum in winter and minimum during the monsoon season. The authors also observed an elevated concentration of PAHs in winter, probably due to the increased consumption of fossil fuel, primarily used for the heating purposes. On average, the annual concentrations of ∑PAHs at three sites in Delhi (India), (i.e. Okhla, Daryaganj and Dhaulakuan) were 1049.3 ng m^{-3}, 1344.3 ng m^{-3}, and 1117 ng m^{-3}, respectively, in which, the level of high molecular weight (HMW) PAHs was highest (i.e. 88.14 %). According to the authors (Sharma et al. 2007), an increase in PAHs concentrations at Daryaganj was due to the diesel and gasoline driven vehicles and coal combustion sources.

In another study (Masih et al. 2012), the concentration of $\sum_{23}$PAHs (gaseous and particulate phase) in the outdoor air of rural Indian homes (North central part), was observed in the range of 21.9 and 1290 ng m^{-3}. Furthermore, the concentrations were high in winter, low in summer (11.2–613 ng m^{-3}) and least during the rainy season (13.1–272 ng m^{-3}). Gas-phase PAHs were dominated by 2,4-rings PAHs, while the Naphthalene (Nap) was the most abundant congener in PAH profile, and 5,6-rings congeners were predominated in all sampling locations. The annual mean concentration of particles-bound (<10 μm) Σ16PAHs (74.7 ng m^{-3}) in Delhi, India was dominated by 4,6-rings compounds (i.e. ~85 %) (Sarkar and Khillare 2011). A similar trend was also observed in Nepal where the concentrations of gas-phase and particulate bound-PAHs (PM_{10}) were associated with the combustion of coal in

Table 1 Summary of mean concentrations of PAHs in different environmental matrices of South Asia

	Study Area	Sampling year/ month	Analytical Methods (Extraction, Analysis)		Nap	Acy	Ace	Fl	Phe	Ant	Fla	Pyr	B(a)A	Chry	B(b)F	B(k)F	B(a)P	IP	DB	BghiP	∑PAHs	Reference
Atmospheric (ng m^{-3})	Karachia		Ultrasonic extraction	GC-MS	–	–	–	–	–	–	11.4	21.4	18.5	–	–		–		–	–	51.3 (particulate phase)	Padma et al. (2007)
	Karodia		Ultrasonic extraction	GC-MS	–	–	–	–	–	–	2.45	3.64	29.9	–	–		–		–	–	36.0 (particulate phase)	
	Koyalia		Ultrasonic extraction	GC-MS	–	–	–	–	–	–	4.05	17.6	79.1	–	–		–		–	–	100 (particulate phase)	
	Bajawa		Ultrasonic extraction	GC-MS	–	–	–	–	–	–	13.7	13.28	14.8	–	–		–		–	–	41.8 (particulate phase)	
	Undera		Ultrasonic extraction	GC-MS	–	–	–	–	–	–	16.2	9.1	–	–	–		–		–	–	25.3 (particulate phase)	
	Tapti		Ultrasonic extraction	GC-MS	–	–	–	0.43	1.29	1.53	5.39	15.41	–	–	4.07		–		–	–	35.4 (particulate phase)	
	Road side (Agra)	Winter	Soxhlet extraction	GC-MS	1314	174		71.1	110	17.9	33.6	29.16	31.3	–	35.6		27.9		0.3		1757 (vapor + particulate phase)	Manish et al. (2010)
	Urban (Agra)	Winter	Soxhlet extraction	GC-MS	562	25.5		24.9	29.9	43.6	10.9	14.9	–	–	–		6.5				686 (vapor + particulate phase)	
	Nunhai (Agra)	Dec 2005–2006	Ultrasonication	GC-MS	–	3.6		6.7	6.5	2.8	6.7	4.6	29.4	101	11		–		20.4	10.5	81.6 (Particulate phase)	Rajput and Lakhani (2010)
	Kathmandu Valley	Winter, 2003	Soxhlet extraction	HRGC/ HRMS	–	–		13	0.09	0.58	–	18	16	–	–		20		2	–	15.6 (vapor + particulate phase)	Kishida et al. (2009)
	Industrial Side (Lahore)	Aug–Sep 1992	Ultrasonication	HPLC	0.43	2.12	2.12	0.73	14.1	4.6	2.24	2.33	4.92	6.67	7.7		7.93		3.01	11.0	39.5 (Particulate phase)	Smith et al. (1996)
	Rural side (Lahore)	Aug–Sep 1992	Ultrasonication	HPLC	0.45	3.08	3.08	1.17	1.16	2.93	2.53	2.54	5.85	8.5	10.8		10.1		4.32	13.2	48.7 (Particulate phase)	
	Urban side (Lahore)	Aug–Sep 1992	Ultrasonication	HPLC	0.39	2.78	2.78	0.97	0.97	4.99	2.81	2.93	5.39	8.64	9.8		9.32		3.85	14.3	49.4 (Particulate phase)	

	Urban area (Kandy)	May–Aug 2009	Soxhlet extraction	HPLC	5.56	3.03	1.02	0.08	0.03	0.01	0.08	0.08	0.03	0.005	0.03		0.22		0.005	0.01	10.0 (Particulate phase)	Karunaratne and Wijayarathna (2013)
	Industrial (Agra)	May 2006–Dec 2009	Ultrasonication	GC	0.91	1.73	1.82	1	0.9	2.2	0.9	3.1	–	–	–		12.3		–	11.1	31.9 (Particulate phase)	Likhani (2011)
	Urban (Delhi)	Jan 2002–Dec 2003	Ultrasonication	GC	–	–	–	–	30.6	27.8	67.4	61.2	–	–	93.8		21.7		90.3	100	364 (Particulate phase)	Sharma et al. (2007)
Water (ng L^{-1})	Bangsai River, Bangladesh	Aug, 2009	Liquid-liquid extraction	GC-MS	2E+06	–	–	3E+05	ND	90,000	–	–	–	–	–	–	–	–	–	–	2,380,000	Hossain et al. (2011)
	Buriganga River, Bangladesh	July, 2000	Liquid-liquid extraction	GC-MS	–	–	–	35,600	–	19,750	–	37,150	–	14,550	–	–	–	–	–	–	123,704	Motaaleb et al. (2003)
	Gomti River, India	Dec, 2002	Liquid-liquid extraction	HPLC	1273	11,707	–	–	300	10	450	100	–	–	310	600	760	324	1933	–	15,500	Malik et al. (2004)
	Yamuna River, India		Liquid-liquid extraction	HPLC	ND	–	ND	–	3510	ND	4610	ND	–	–	–	–	–	–	–	–	8120	Shabeer et al. (2013)
	Harbor Line, Mumbai, India	2008	Liquid-liquid extraction	HPLC	90	600	940	566	1080	90	1300	90	1440	90	5700	880	1410	5120	5440	3530	22,680	Dhananjayan et al. (2012)
	Gomti River, India		Liquid-liquid extraction	HPLC	910	7946	–	–	190	70	190	50	–	–	22	70	110	–	170	–	10,662	Malik et al. (2011)
	Chenab River, Pakistan	2007–2009	Liquid-liquid extraction	GC-MS	215	53.7	47.5	94	166	36	36	64	1.4	3.1	2	2	1	0.2	0.05	1.35	669	Farooq et al. (2011)
	Rawalpindi, Pakistan		Liquid-liquid extraction	GC-MS	28.6	–	148	–	179.2	179	168	–	–	71.2	–	–	–	–	–	–	775	Saba et al. (2012)
	Soan River, Pakistan	2013	Liquid-liquid extraction	GC-MS	22.4	20.4	12.4	10.7	3.2	17.7	10.1	7.6	2.5	2.8	2.5	10.5	2.8	2.4	1.7	3.1	134.4	Aziz et al. (2014)
	Beira Lake, Sri Lanka	May–Sep	Liquid-liquid extraction	GC-MS	–	–	–	–	–	–	–	106		–		–	–	–	–	–	154	Pathiratne et al. (2007)
	Bolgoda lake, Sri Lanka	May–Sep	Liquid-liquid extraction	GC-MS	–	–	–	–	–	–	–	90	–	–	–	–	–	–	–	–	145	
	Sagarmatha National Park, Nepal	Oct 2007	Liquid-liquid extraction	GC-MS	–	0.03	0.07	0.152	0.62	ND	0.26	0.05	ND	ND	ND	ND	ND	–	–	–	1.9	Guzzella et al. (2011)

(continued)

Table 1 (continued)

	Study Area	Sampling year/ month	Analytical Methods (Extraction, Analysis)		Nap	Acy	Ace	Fl	Phe	Ant	Fla	Pyr	B(a)A	Chry	B(b)F	B(k)F	B(a)P	IP	DB	BghiP	∑PAHs	Reference
Soil/Sediments (ng g^{-1})	Islamabad		Ultrasonication	HPLC	8.46	–	–	–	6.47	–	–	5.54	–	–	–	–	6.02	5.91	–	–	5.91	Din et al. (2013)
	Rawalpindi		Ultrasonication	HPLC	7.38	–	–	–	4.21	–	–	8.33	–	–	–	–	5.01	5.02	–	–	5.02	
	Delhi	Jan, 2006	Ultrasonication	HPLC	0.24	0.18	0.28	0.07	0.07	0.04	0.19	0.12	0.12	0.02	0.08	0.05	0.04	0.07	0.13	0.09	10.9	Agarwal et al. (2009)
	Industrial (Agra)		Ultrasonication	HPLC	1.18	0.62	–	–	0.43	1.29	1.72		0.81	–	–	–	–	–	–	1.01	11.0	Masih and Taneja (2006)
	Roadside (Agra)		Ultrasonication	HPLC	1.04	0.47	–	–	0.32	1.02	1.29	1.23	0.56	–	–	–	0.39	–	–	0.85	12.0	
	Residential (Agra)		Ultrasonication	HPLC	0.92	0.33	–	–	0.41	0.57	0.89		0.45	–	–	–	–	–	–	0.64	13.0	
	Agricultural (Agra)		Ultrasonication	HPLC	0.69	0.42	–	–	6.14	0.36	0.58		0.26	–	–	–	–	–	–	0.54	13.8	
	Delhi (Airport)	Nov 2005–May 2006	Ultrasonication	HPLC	–	–	–	–	0.27	0.25	0.71	0.78	0.17	0.14	0.28	0.31	0.22	0.19	0.09	0.36	14.4	Ray et al. (2008)
	Sewri		Soxhlet extraction	HPLC	–	–	0.11		0.12	0.11	0.01	0.08	0.02	0.08	0.93	0.20	0.09	0.55	0.13	0.33	14.6	Dhananjayan et al. (2012)
	Mahul		Soxhlet extraction	HPLC	–	–	18.2	0.09	0.03	0.02	24.8	0.27	0.05	0.17	0.01	17.4	0.20	0.55	0.99	0.51	15.2	
	Nahva		Soxhlet extraction	HPLC	–	–	–	0.01	0.41	0.65	0.01	0.08	0.15	0.19	0.29	0.09	0.73	–	0.21	12.5	15.7	
	Gomti River	March 2004–Feb 2006	Soxhlet extraction	HPLC	0.05	0.25	0.08	0.11	0.07	0.01	0.02	0.01	–	–	0.01	0.05	0.03	–	0.09	–	–	Malik et al. (2011)
	Yamuna River		Ultrasonication	HPLC	3.54	0.58	–	–	0.27	0.55	0.72	1.25	1.16	0.73	0.06	0.36	0.12	0.05	0.11	0.15	–	Agarwal et al. (2006)

Abbreviation: Napthalene (Nap), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Flu), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Fua), Pyrene (Pyr), Benzo (a)anthracene (BaA), Chrysene (Chr), Benzo (b)fluoranthene (BbF), Benzo(k)fluoranthene (BkF), Benzo(a)pyrene (BaP), Indeno(1,2,3-cd)pyrene (IP), Dibenz(a,h)anthracene (DiB), Benzo(g,h,i)perylene (Bghip). ND (Not detected); 1(Summer), 2(Monsoon), 3(Winter)

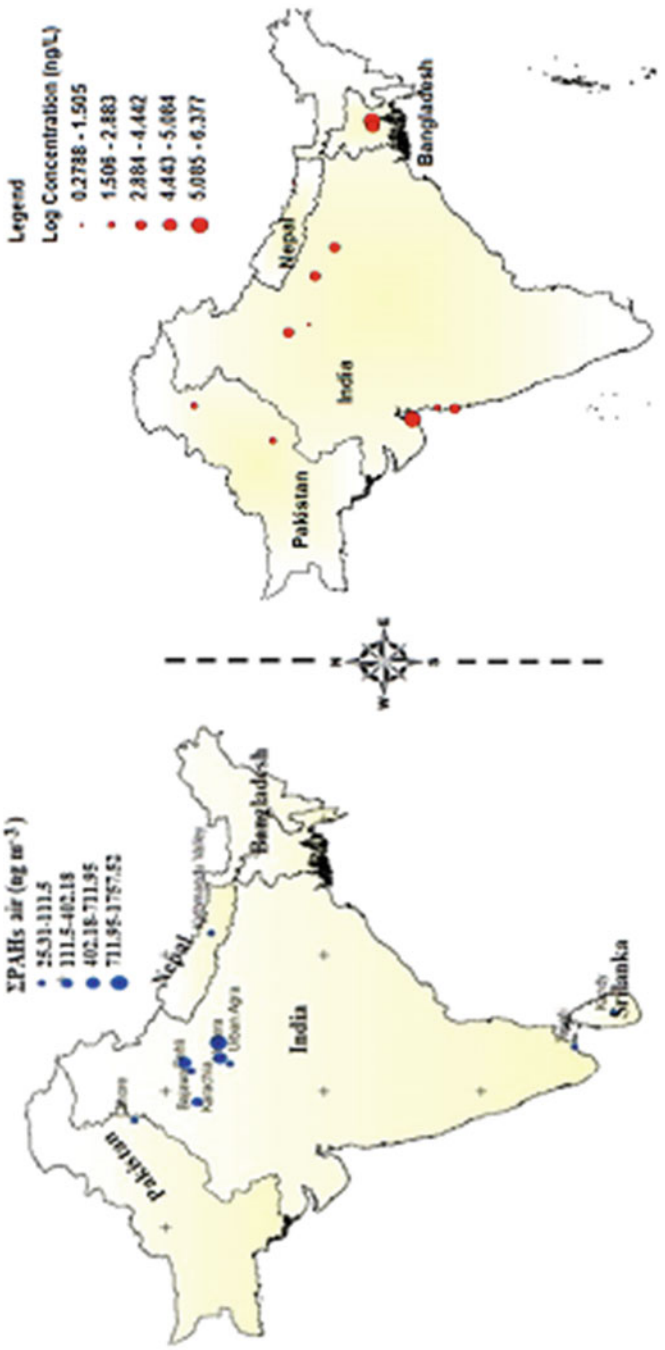

Fig. 2 Spatial distribution of ∑PAHs in air and water bodies of South Asia

brick kilns in the Kathmandu Valley. The mean concentrations of $\sum_{45}$PAHs in December 2005 were 210 ng m^{-3} and 430 ng m^{-3} in the total PM- and gas-phase, respectively. PAHs profile was also dominated by 4,7-rings congeners (80.38 %), whereas Phe was the dominant PAH in the gas-phase (Kishida et al. 2009).

A comparative study of PAHs distribution in indoor and outdoor environments (urban and roadside homes) in Agra (India) showed that the average concentrations of PAHs in roadside homes (i.e. 1846.58 ng m^{-3}) in the indoor and outdoor air samples were higher than those in the urban homes (718.45 ng m^{-3}) due to the influence of vehicular emissions (Masih et al. 2010). Nap was dominant in all indoor and outdoor environments and ranged from 562 to 1315 ng m^{-3}. Spatially, the ∑PAHs in the indoor environment were found in higher concentration in the kitchen areas than in the living rooms. In fact, combustion of liquefied petroleum gas (LPG) for cooking purposes during frying, stir frying, deep frying, boiling, steaming and stewing food are common, which may be responsible for generating PAHs in the kitchen environment (Zhu et al. 2001). In a study conducted by Rao et al. (2007), Benzo(a)anthracene B(a)A was detected at high levels due to a nearby refinery processes, such as catalytic cracking, distillation, storage and fuel combustion. ∑PAHs concentrations detected in ambient aerosols in Mumbai, Ahmadabad, Kolkata, Nagpur, and Kanpur were found to be 10–50 times higher than those reported internationally and ranged between 23.0 and 190 ng m^{-3} (Kulkarni and Chandra 2000).

The concentration of particulate-bound PAHs in the urban areas is largely influenced by the degree of urbanization, types of fuel used, and proximity to road. These factors were influential on the increased indoor-PAHs of Kandy City (Sri Lanka) where the fuel wood was used for cooking purposes in the indoor environment (Karunaratne and Wijayarathna 2013). Other PAHs sources included biomass combustion and vehicular emissions due to proximity to the roads. They observed a PAH concentrations in the range of 0.39 and 14.6 ng m^3. The most dominated PAH determined was Nap, which accounted for ~43.5 % of the total PAHs. The Nap emissions in particular in the indoor environment were highly correlated with the indoor use of mothballs, combustion of kerosene, and other fuel vapors (Lu et al. 2008).

In Pakistan, there is only a preliminary study available, which reports PAHs levels in air samples at industrial and rural sites in Lahore city in mid 1990s (Smith et al. 1996). The average ΣPAHs ranged between 0.39 and 14.6 ng m^{-3} in the urban areas. Benzo(ghi)perylene B(ghi)P, markers vehicular emissions, was the most abundant congener of PAHs. High concentrations of PAHs (120 ng m^{-3}) were observed in rural site where brick kilns were the main source attributed to elevated levels of PAHs pollution. In fact, the regional climatic conditions could also greatly influence the concentrations of PAHs present in airborne particles. However, the consumption of fossil fuels, emissions from brick kilns and low dispersion in winter remained the major sources.

2.2 Water Bodies

PAHs enter water bodies through the atmospheric deposition and direct release of substances through petroleum spills and use, municipal wastewater treatment plants, industrial discharges, storm-water runoff, landfill leachate, and surface run-off. It has been estimated that 10–80 % of PAHs input to the world's oceans is from atmospheric sources (Manoli et al. 2000). Chen et al. (2004), estimated that the atmospheric PAHs deposition in the water bodies in the area of Hangzhou city (China) was 530 t $year^{-1}$, while the contribution of surface runoff water was 30.7 t $year^{-1}$. As a consequence of their hydrophobic nature, PAHs in aquatic environments rapidly tend to become associated to the particulate matter (PM) ending in sedimentation. In general, a comparative analysis revealed that the level of pollution in the surface waters was highest in Bangladesh followed by the India > Pakistan > Sri Lanka > Nepal (Fig. 2; Table 1). ∑PAHs concentration levels in the SAR ranged between 1.9 ng L^{-1} (Himalayan Lakes, Nepal) and 2,380,000 ng L^{-1} (Bangsai River of Bangladesh). The Bangsai and Buriganga Rivers of Bangladesh were found to be the most contaminated with an average PAHs concentration of 2,380,000 ng L^{-1} and 123,704 ng L^{-1}, respectively (Hossain et al. 2011; Motaaleb et al. 2003). Both rivers flow through Dhaka, the capital city and have a massive effect on the socio-economic development of the country by shipping activities. The sewage from the tanker washing, ship scrapping and oil spills could be potential sources of PAHs in the water bodies. In addition, the disposal of industrial waste into surface water introduces huge amounts of aromatic solvents, resulting in a consequent increase in PAHs pollution. This could be a sound reason behind high concentration of Nap in the Bangsai River (2×10^6 ng L^{-1}).

Water bodies of Himalayas have a fundamental role for the dependency of 500 million people living nearby the basins of Indus, Brahmaputra and Ganges Rivers (Salerno et al. 2008). Thin layers of soil, sparse vegetation cover, dense population, intense agriculture and increased industrial activity in the adjacent areas are assumed to be the prominent sources of PAHs in the Himalayan lakes. Guzzella et al. (2011), published the first data on PAHs in the Nepalese region of Sagarmatha National Park and confirmed that the Himalayas are an undisturbed and remote area. Low molecular weight (LMW-PAHs) were found in water samples, confirming the hypothesis that the lighter compounds are more prone to LRAT, whereas five or more aromatic rings PAHs were often not detected.

Industrialization and urbanization have escalated rapidly during the last few decades in India. As compared to other countries of the South Asia, many studies have documented the surface water contamination of PAHs in the Indian region. The Indian aquatic water bodies receive huge amounts of contaminants from sewage waste water, industrial effluents, and urban or rural run-off. Shabeer et al. (2013), investigated PAHs levels in a tributary water of Ganga, the Yamuna Rivers. PAHs were not detected in the water bodies of Wazirabad and Aakulam areas, however the water sample collected from Yamuna river at Okhla region

contained residues of Phenanthrene (Phe; 3510 ng L^{-1}) and Fluoranthene (Fla; 4610 ng L^{-1}). The contamination could be ascribed to the dumping of untreated industrial waste into the river.

Dhananjayan et al. (2012), have reported the PAHs occurrence in water and sediment samples collected from the Harbor line Mumbai (India). PAHs related with the contamination from the sewage discharges, industrial wastewater, urban run-offs and intense shipping and oil refinery activities. Among the individual PAH-congeners, Benzo(b)flouranthene B(b)F (i.e. HMW PAHs) was dominant.

According to Mailk et al. (2011), the temporal and spatial distribution of $\sum_{16}$PAHs in Gomti River ranged from 60 to 84,210 ng L^{-1}, with an average value of 10,330 ng L^{-1}, which were noted much higher than the guideline value (i.e. 0.20 μg L^{-1}) prescribed by the Bureau of Indian Standards. LMW-PAHs including Phe and Nap were found dominant. The authors mentioned that maximum PAHs were observed during the low rate of the River discharge. However, lower concentrations during the monsoon season were detected which were possibly due to the heavy rainfall and it could pose dilution impact on the river flow that leads to the dilution of the total concentrations of contaminants (Doong and Lin 2004). The authors concluded that Gomti River is highly polluted and poses high risk to the aquatic life. In another study by Pandit et al. (2006), the PAHs in the surface water of creek adjoining the Mumbai harbor (India) ranged between 84.3 and 377 ng L^{-1}, with an average of 182.1 ng L^{-1}. The dismantling of ships is one of the businesses which generate huge amounts of petroleum hydrocarbons; these pollutants are gradually accumulated in the soil and reach the sea water through tides and sub-tides. In India, Alang-Sosiya ship scrapping yard receives around 70 % of the world's ships for various services or dismantling. In this context, Srinivasa et al. (2005) have made some evaluation of seasonal PAHs in the surface water of Alang–Sosiya during high tides. They observed high PAHs concentrations in particular; the highest PAHs concentrations were detected in the winter season followed by the monsoon and summer seasons. High PAHs concentrations during winter season were attributed to the water-residence time which could be influential on the accumulation and retention of hydrocarbons in the seawater. The dilution factor in monsoon could also be responsible for lower concentrations. High temperature and low atmospheric pressure during summer could account for the increased volatilization of PAHs, which resulted in low concentrations in summer.

The spatial and seasonal distribution of PAHs in the aquatic system of River Chenab (Pakistan) was reported by Farooq et al. (2011), in this case, the predominant PAHs in the surface water of River Chenab were Nap (215 ng L^{-1}) and Phe (166 ng L^{-1}). As the seasonal trend observed in the previous studies, the PAH concentrations were relatively higher in winter than in summer. The industrial activities, coal/trash burning in agricultural areas and the disposal of the municipal waste from surrounding urban and sub-urban areas directly into the river ecosystem could be the principal sources of PAHs. Comparing to other regions of the world, the PAHs concentrations in the River Chenab were considered under moderate risk.

PAHs contamination is also influenced by the population density along the catchment areas of the water bodies. This phenomenon was investigated by

Pathiratne et al. (2007) in the Bolgoda and Beira Lakes (Sri Lanka), which were selected for the study due to their economic value and high level of pollution. The concentrations of PAHs in these lakes ranged from 46 to 112 ng L^{-1} in Beira and from 40 to 127 ng L^{-1} in Bolgoda Lake. The PAHs congeners with 4,5-rings ranged from 57 to 66 % of total concentrations in water samples. The level of contamination seemed to be increasing because of the lack of wastewater treatment plants, and the discharge of huge amounts of untreated wastewater directly into the lakes. In addition to these factors, Bolgoda Lake also receives massive amounts of industrial effluents via Ganga River, which is a major tributary that sprouts of the Lady Catherine industrial zone in the north (Dehiwala area). Vehicular emission was also considered as a potential source of PAHs in the lakes.

PAHs in groundwater may also originate from the polluted surface water bodies, agricultural irrigation with effluents, leachates from solid waste disposal sites and/or contaminated soils. It has been established that the disinfection of drinking water could result in the synthesis of oxygenated and chlorinated PAHs, i.e. compounds more toxic than the parent PAHs (Manoli and Samara 1999). Such contamination of ground water resource poses a substantial risk to local resource user and to the natural environment. To better of our knowledge, only a few studies are present in literature focusing on the occurrence and sources of PAHs in groundwater in South Asia. In Pakistan, Saba et al. (2012) investigated the distribution, and toxicity of $\sum_6$PAHs in groundwater of Rawalpindi and found that PAHs levels were higher than the recommended limit (0.2 μg L^{-1}) of PAHs for drinking water established by WHO (WHO and IPCS 1998). Ant, Phe and Fl, which contributed 61 %, 5.0 %, and 4.6 % to ∑PAHs, respectively, were the predominant congeners. The presence of LMW-PAHs suggested their association with organic content, reducing loses by volatilization. Authors evidenced that the main sources were atmospheric emission, combustion products of PAHs from gas flaring and vehicular exhaust.

2.3 Soil/Sediments

The surface soil/dust/sediment is an important sink for several different hydrophobic organic contaminants such as PAHs (Ockenden et al. 2003; Cai et al. 2008; Kamal et al. 2015; Tuyen et al. 2014). These soil/dust-bound PAHs pose potential risk to human health (Kamal et al. 2015) and also lead towards the cellular toxicity to both plants and microorganisms (Menzie et al. 1992). PAHs concentrations in the soils/dust and sediments from different parts of the South Asian countries have been summarized in Table 1. In fact, the information on the concentration and distribution of PAHs in soils is not sufficiently available in the South Asian countries, such as Pakistan, India and Nepal etc. In general, PAHs contamination levels in the soil/sediments were higher in India than Sri Lanka and Pakistan (Fig. 3). In India, soil contamination by PAHs is of great interest, because urban and suburban soils in Delhi city are extensively used for agricultural purposes, especially vegetable

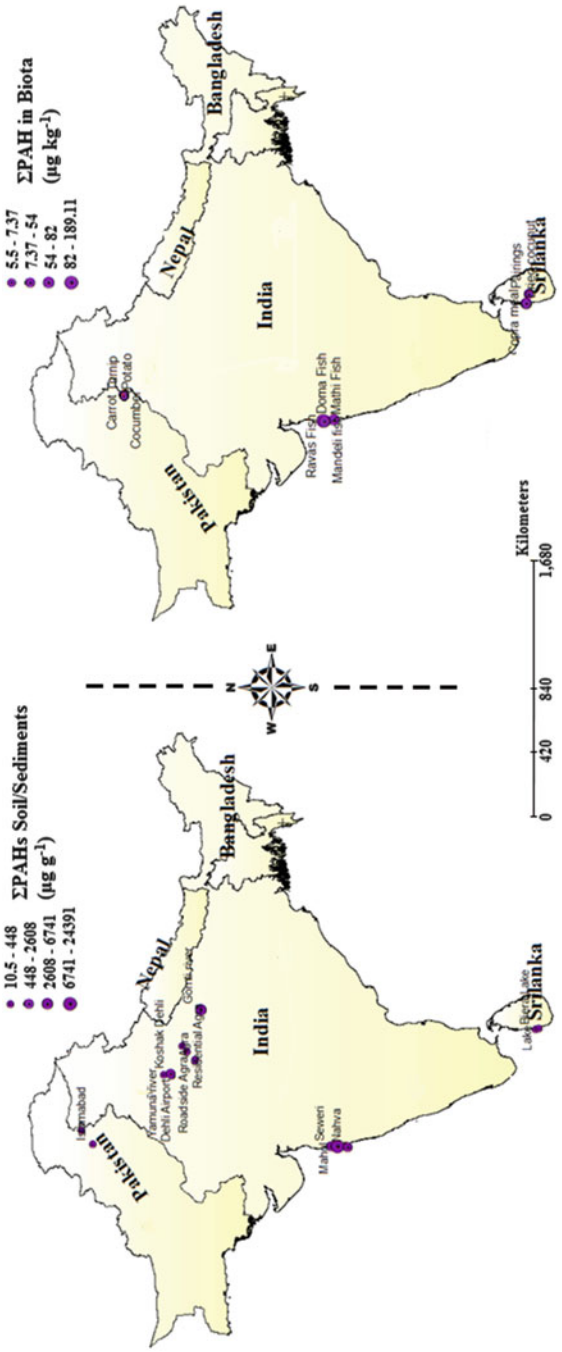

Fig. 3 Spatial distribution of ∑PAHs in soil/sediments/biota of South Asia

production and posing a health risk to human population. According to a study conducted by Agarwal et al. (2006), the concentrations of $\sum_{16}$PAHs in the soils of different agricultural sites, were 2–5 times higher in urban agricultural soils than those in rural sites.

In general, LMW-PAHs dominated the PAH profile in the agricultural soils of Delhi which reflected the significant combustion emission from the low temperature pyrolytic processes. Another study conducted by Masih and Taneja (2006), in the industrial, residential, roadside and agricultural areas showed that an average concentration of $\sum_{14}$PAHs (12.1 μg g^{-1}) in all samples and the range was from 3.10 to 28.5 μg g^{-1}. In these samples, presence of high concentration of Chry and B(b)F suggested the combustion sources, including oil burning, wood combustion and emissions from diesel powered vehicles (Ravindra et al. 2007). In addition, the surface soil in the industrial sites had the highest levels of the $\sum_{12}$PAHs, which could be originating from the local tanning industries, generator manufacturing and iron casting processes. Similarly, the soil samples of international airport in Delhi (Ray et al. 2008) also had high concentrations of PAHs (i.e. ranging between 2.39 and 7.53 μg g^{-1}). In these sites, Pyr (19 %) and Fla, DBA (16 %) were the most abundant PAHs congeners. PAHs concentrations at this airport could be originating from the incineration process, oil combustion and high temperature condensation of low molecular weight PAHs (Soclo et al. 2000).

In Pakistan, the presence of petrol pump stations, vehicular emissions and burning of fossil fuel have been suggested as important contamination sources in Islamabad and Rawalpindi cities (Din et al. 2013). Nap, which can be considered a marker of spilled oil and petroleum products was found in concentrations ranging from 2.47 to 24.36 μg g^{-1} (Kamal et al. 2011). Some recent studies have reported the concentrations of 18-PAHs in different regions of the Punjab Province (Pakistan). Results revealed that higher concentrations of PAHs were found in the soil/dust samples of brick kiln units of Sohdra town (302–6757 ng g^{-1}), Gujranwala city (16–1963 ng g^{-1}), Chung (692–1007 ng g^{-1}) followed by the roadside soil/dust samples of Lahore city (ranging between 357 and 4530 ng g^{-1}), soil/dust samples from auto-mechanics shops in Rawalpindi city (ranging between 690 and 1496 ng g^{-1}), and dust samples from the petroleum refinery situated in Rawalpindi (692 and 1004 ng g^{-1}) (Kamal et al. 2014). This study provides evidences that PAHs pollution in different cities of Pakistan was strongly influenced by anthropogenic activities and industrial sector, in particular brick kiln and petrochemical industries.

Aichner et al. (2007), investigated surface soils collected in Kathmandu (Nepal) and found Σ_{20}PAHs concentrations ranging between 0.18 and 10.2 μg g^{-1}. The most abundant PAHs included Pyr (14.6 %), B(bjk)F (10.7 %), Nap (10.7 %) and Phe (9.8 %). High Pyr concentrations indicated biological sources, probably due to anaerobic degradation of perylene-quinones. In general, sediment cores are useful in rebuilding historical records and evaluating the contaminants present in the environment (Lima et al. 2003). Many studies have been conducted to investigate the sedimentary records of PAHs in different South Asian regions. Surface sediments from ten different stations located down the stretch of the Hugli estuary

showed $\sum_{17}$PAHs (2, methylated Napthalene) and $\sum_{16}$EPA priority PAHs with an average concentration of 6.40 ng g^{-1} while 4,5-rings PAHs, such as Phe, Pyr, BbjkF, and Chry were the dominant congeners. Results highlighted that anthropogenic activities like domestic and industrial discharges were the main sources of contamination. Average $\sum_{15}$PAHs concentrations in the sediments along the harbor line Mumbai (India) (Dhananjayan et al. 2012). ranged between 8.66 and 46.7 μg L^{-1}, dominated by 4-rings PAHs which might be released due to pyrolysis of fossil fuels. High concentrations of PAHs found in the sediments could be attributed to the large amount of soil run-off and sewage discharged from this area into seawater.

2.4 Biota

Bioaccumulation of PAHs in the adipose tissues of organisms varies considerably and it is influenced by the nature of individual PAHs, the extent of exposures and the capability of the organism to metabolize these contaminants (Lü et al. 2014). Humans are largely exposed to environmental contaminants from three main pathways, i.e. inhalation, ingestion and dermal contact in both occupational and non-occupational settings (Ravindra et al. 2008). In petrochemical workplaces, humans may be exposed to PAHs especially during the process of oil refining, mining, repairing the motor vehicle etc. (Karthikeyan and Balasubramanian 2006). Moreover, the work related parameters, such as exposure time, also influence the extent of exposure to PAHs. In addition, concentration of PAHs, related toxicity, route of exposure, workers pre-existing health conditions and age can also influence the endpoint of human exposure (Unwin et al. 2006).

The vegetation may be also contaminated with PAHs from the deposition of gas phase PAHs, and/or by the uptake of soil-laden PAHs (Lü et al. 2014). There are few studies reporting the occurrence and distribution of PAHs in vegetation of South Asia (Table 2). In Pakistan, Ashraf et al. (2013) evaluated the distribution and associated risks of PAHs in vegetables in Punjab Province. Authors observed that the underground vegetables e.g. potato and carrot had highest concentration of PAHs (average 13 μg kg^{-1}) compared with turnip (10.9 μg kg^{-1}). Additionally, the fruits skin had more PAHs contents than the core of different fruits. The concentration of Ant dominated the PAHs profile in all the cases. The concentration of PAHs could be associated with local anthropogenic activities. In Delhi (India), vegetables grown in the vicinity of a power plant, showed high concentrations of Phe, Ant, Fla, Pyr and Chry (Khillare et al. 2012). It was also observed that the concentration of PAHs in vegetable was low due to background site exposure (ranged from 2.0 to 500 μg kg^{-1}) compared to those grown near the power plant (ranging from 70.0 to 1100 μg kg^{-1}). Dhananjayan and Muralidharan (2012) investigated PAHs in five species of fish samples along the harbor line of Mumbai, India. Total PAHs concentration ranged from 17.4 to 70.4 ng g^{-1} among which Mandeli, *Coilia dussimieri* contributed highest intakes PAHs followed by Doma,

Table 2 Summary of mean concentrations of polycyclic aromatic hydrocarbons in food (ng g^{-1}) of South Asia

Location		Flu	Pyr	B(a)A	B(b)F	B(a)P	Nap	Acy	Fl	Phen	Ant	B(a)f	Chry	B(k)f	B(ghi)p	D(a,h)	∑PAHs	Reference
Copra	Colombo	186	137	82	19	8	–	–	–	–	–	–	–	5	1.7	2	440.7	Wijeratne et al. (1996)
Coconut oil		31	24	7	1	1	–	–	–	–	–	–	–	–	–	–	64	
Copra meal		5	1	8	7	55	–	–	–	–	–	–		4.8	ND	2	82.8	
Dried Coconut		3	2	4	5	15	–	–	–	–	–	–	–	0.7	–	–	29.7	
Pairings		8	4	5	18	17	–	–	–	–	–	–	–	0.5	ND	0.9	53.4	
Doma Fish	India	2.17	1.64	7	34	6	19.94	2.98	5.36	0.61	ND	–	ND	–	–	–	79.7	Dhananjayan and Muralidharan (2012)
Mandeli Fish		2.1	ND	76	54	66	43.14	5.48	4.84	0.99	ND	3.71	8.85	ND	–	–	265.11	
Mathi Fish		3.2	ND	54	43		17.67	1.3	4.1	ND	ND	ND	ND	3.54	1.99	–	128.8	
Ravas Fish		5.2	ND	23	25	1.25	14.04	6.13	ND	ND	1.89	3.46	2.79	ND	2.31	–	85.07	
Potato	Lahore	–	–	–	–	–	1.75	–	–	–	–	2.88	1.18	–	1.12	–	6.93	Ashraf et al. (2013)
Turnip		–	–	–	–	1.19	–	–	–	–	–	2.74	–	2.45	ND	–	6.38	
Carrot		–	–	–	–	3.17	–	–	–	–	–	2.1	–	2.1	ND	–	7.37	
Cucumber		–	–	–	–	2.35	–	–	–	–	–	2.5	–	0.97	0.78	–	6.6	
Bitter ground		–	–	–	–	1.33	–	–	–	–	–	0.76	–	0.67	ND	–	2.76	

Otolithes ruber. Authors highlighted that higher concentrations of PAHs might be associated with the consumption rate, accumulation level and the availability of fish.

Another study by Wijeratne et al. (1996), from Sri Lanka determined the concentration of PAHs in the edible coconut kernel products. The average $\sum_8$PAHs concentration in the items such as copra, coconut oil, copra meal and parings was found to be in the concentration of 102 μg kg^{-1}, 359 μg kg^{-1}, 68 μg kg^{-1} and 109 μg kg^{-1} respectively. The PAHs concentration in the coconut kernel products might be originating from the process of combustion of shells in controlled conditions.

3 Regional Comparison

3.1 Atmospheric PAHs

There are evident differences in PAHs emission and their source among different regions. In fact, China is the largest contributor to the atmospheric PAHs in the world (Inomata et al. 2013). According to Zhang and Tao (2009), the China accounts for highest emissions of PAH which is roughly equal to 114 Gg $year^{-1}$, followed by the India (90 Gg $year^{-1}$) and United States (32 Gg $year^{-1}$). The emission of PAHs is significantly influenced by the population density, energy composition and economic development of the region (Kamal et al. 2015). For instance in the United States, the consumer products were the major sources of exposure among communities. Nevertheless, there was also a declining trend in the PAHs emission from the Europe, after a substantial decline in domestic consumption of fossil fuels. It has been reported that over the 5 year there was around 30 % declined per annum in the total PAHs concentrations (Katsoyiannis et al. 2011). In another evaluation, the atmospheric PAHs concentration during two decades (1990–2010), there were changes in combustion types and sources over times reduces the PAHs levels (5500 t $year^{-1}$ in 1990 to 600 t $year^{-1}$ in 2009) respectively. The evaluation was based on the source inventory dataset from different counties of UK including Manchester, London, Liverpool, Birmingham, Middleborough, and Edinburgh. UK ambient air monitoring program include a wide range of rural, urban and semi-rural units. The study revealed that at the end of nineteenth century there was a rapid increase in the industrial development. Consequently, the concentration of PAHs reached its maximum levels (32 ng kg^{-1}) owing to the emission of gasoline and diesel engines compared to that from coal and wood burning used in early 1860–1980s (Gabrieli et al. 2010). Likewise, the spatial and seasonal differences of atmospheric PAHs were influenced by the exhaust emission of petrochemicals, iron steel plants in the Turkey (Kaya et al. 2012). Furthermore, in another European region, the $PM_{2.5}$ associated PAHs in Tuscany, (Italy) ranged from 0.92 to 13 ng m^{-3} with a two to threefold decline in the

PAH-associated cancer risk as compared to that late 1990s (Martellini et al. 2012). Status of PAHs in urban and industrial environment of Prato (Italy) was determined by Cincinelli et al. (2007) and they observed that $\sum_{12}$PAHs in both particulate and gas-phases (59.4 ng m^{-3}) were related to the strong influence of diesel fuel burning. Authors further highlighted that the abundance of PAHs could be influence by the climate, for instance, the concentration was inversely proportional to the rise in temperature. Similar results of PM_{10}-bound PAHs were observed in another study conducted in Prato, (Italy) In which case, the concentration of the $\sum_{16}$PAHs ranged from 3058 to 22,048 ng m^{-3} (Cincinelli et al. 2004). Another study from Brazil also provided some information on PAHs levels, in which case, the PAHs congeners were emerging mainly from the emission of wild fire, whereas the mobile sources were also the main pollutant emitters in the Latin America (Fernandes et al. 2002).

In developing countries of the South Asia, there are similar but large differences in the usage of fuels, due to which the combustion emission and the associated factors dramatically vary in this regions. For instance, a high levels of PAHs are released from the vehicular and domestic emissions of PAHs, from India (Sharma et al. 2007), brick kiln in Nepal (Kishida et al. 2009). Likewise in Pakistan, Sri Lanka, Maldives projected increase in the use of gasoline and diesel powered automobiles are among the major sources of PAHs emission.

3.2 Region Abundance of PAHs in the Water Bodies

In general, the overall comparison of PAHs concentration in waters bodies of South Asia with the literature studies from other parts of the world seems quite difficult, because of varying number of PAHs congeners analyzed, and the temporal difference in analysis. Nevertheless, taking into consideration the available literature, the concentration of PAHs were relatively higher in this region as compared to that of Raba River (Hungary) (Nagy et al. 2013), Lower Brisbane River, (Australia) (Shaw et al. 2004) and York River, United States (Countway et al. 2003). Emissions of PAHs in these developed countries have decreased significantly in the past decade which is attributed to the increased use of refined fuels (Pacyna et al. 2003). On the contrary, level of PAHs in water in India, Bangladesh and Pakistan is still comparable to those of China. This indicated that much fossil fuel had been used in urban vehicle traffic and in South Asia and is facing rapidly increasing demand for energy due to insufficient energy supply.

3.3 Soil/Dust/Sediments

The PAHs concentrations in soil and sediment of South Asia region were quite similar to those reported in other studies. In some cases, the concentration of $\sum_{16}$PAHs were very high, such as in the arable soils of Poland (ranging from

413 to 100,043 ng g^{-1}; (Maliszewska-Kordybach et al. 2008). and those in the Seine basin, (France i.e. ∑14PAHs ranged from 450 to 5650 ng g^{-1}) (Motelay-Massei et al. 2004). In UK and Norwegian soils, the concentration PAHs ranged between 42 μg kg^{-1} and 11,200 μg kg^{-1} and from 8.6 μg kg^{-1} to 1050 μg kg^{-1}, respectively (Nam et al. 2008). A study conducted by Tuyen et al. (2014), compared PAHs levels in Vietnam and India revealed that in street dust, pyrogenic emission sources were predominant in Vietnam (2520 ng g^{-1}) whereas in India, mixed pyrogenic and petrogenic sources were responsible for raising levels (2200 ng g^{-1}). Another study from Shanghai, China reported high concentration of Σ_{26}PAHs (ranging from 33 to 8650 ng g^{-1}) and that of Σ16PAHs ranging between 83.3 and 7220 ng g^{-1}). In South China the concentration of Σ_{16}PAHs was also quite high, ranging from 160 to 3700 μg kg^{-1}. Some PAH congeners, particularly the Ace, B(b)F, Flu, B(k)F were the most abundant (Wang et al. 2013; Cai et al. 2007), while the concentrations of ∑16PAHs ranging from 65 to 12,000 ng g^{-1} in Korean soil (Kwon and Choi 2013). Sediment samples down the coast of Korea, showed high concentration of ∑16PAHs ranging between 8.8 and 18,500 ng g^{-1} dry weight (Yim et al. 2007). Wilcke and Amelung (2000), examined the presence of PAHs in grassland soils of North America and reported $\sum_{20}$PAHs concentrations ranged from 63 to 321 μg kg^{-1}. Huang et al. (2012) reported the concentration of PAHs in sediments from Zhanjiang Bay ranged between 42.0 and 934 ng g^{-1} and Leizhou Bay from 21.7 to 320 ng g^{-1}. Higher levels may be due to busy sea traffic and intensively industrial activities around the Zhanjiang Bay. $\sum_{15}$priority PAHs concentration in the wetland sediments from Hong Kong ranged between 0.18 and 0.83 μg g^{-1} dried sediment (mudflats) and 0.63–0.96 μg g^{-1} dried sediment (mangroves) (Zheng et al. 2002), while in the sediments samples collected from Toronto, Canada the concentrations of $\sum_{15}$PAHs ranged from 42 to 3300 ng g^{-1} (Wong et al. 2009). Deposition of PAHs is also investigated previously in dated sediment cores from Lakes Nainital and Bhimtal in the Kumaun Himalaya (Choudhary and Routh 2010). In the Lake Nainital, the $\sum_{25}$PAHs concentrations ranged between 12 and 216 μg g^{-1}, with an increasing upwards in the core. A similar profile was observed for the Lake Bhimtal core, where $\sum_{25}$PAHs concentrations ranged between 1.00 and 217 μg g^{-1}. The authors reported low ∑PAHs concentrations in the bottom sediments due to less urban development in early 1990s. An increased concentration of PAHs in fresh sediments was attributed to the anthropogenic activities and forest fires.

4 Source Apportionment Techniques

A number of source and receptor oriented approaches have been developed to estimate quantitative source contribution. The PAH signature with reference to source markers can be determined by various techniques like Molecular Diagnostic

Ratios (MDRs), Principal Component Analysis-Multiple Linear Regression (PCA-MLR), Positive Matrix Factorization (PMF), Monte Carlo Source Apportionment (MCSA), Chemical Balance model (CMB) and Unmix model (Yang et al. 2013). However, each technique has its own advantages and disadvantages therefore, it is generally recommended to apply multiple techniques in order to minimize individual method biasness and thereby strengthen the results (Yang et al. 2013). Yang et al. (2013) compared different source apportionment techniques and found out that other receptor models (PCA-MLR, PMF) don't identify the exact source and make quantitative assessments in terms of the relative contribution of each source. Molecular diagnostic ratios (MDRs) were frequently used because they dilute to a similar extent and distributed equally in the environment (Dickhut et al. 2000). Many researchers demonstrated that the MDRs are likely to fail in the source apportionment of PAHs (Katsoyiannis et al. 2011; Katsoyiannis and Breivik 2014). Author highlighted the complications based on MDRs for atmospheric source apportionment was that individual compounds have different atmospheric residence times and reaction rates therefore by using MDRs where concentrations remains constant between source and receptor makes the results unreliable. These ratios are valid in case very close proximity of the probable sources and receptors. Zhang et al. (2005), suggested that diagnostic ratios of HMW PAHs as more reliable diagnostic indicators compared to LMW PAHs ratios and characterized Ind/(Ind + Bghip) as the most reliable diagnostic ratio. Similarly, MDRs formulas of similar or low vapor pressure were used to curtail the uncertainty induced by volatilization (Cecinato et al. 2014). Among other various techniques Chemical mass balances (CMBs) were used for inorganic and organic compounds. Major demerit of this technique was that it only quantifies particle phase compounds (Larsen and Baker 2003). Callén et al. (2013), highlighted the merit of positive matrix factorization (PMF) with regard to other receptor models was the use of uncertainty matrix that underweight the concentrations below the detection limit. Furthermore, Jang et al. (2013) added that advanced factor analysis tool and the ability to quantify the factor contribution without subsequent use of multiple linear regression (MLR) hence, become the most powerful technique than other factor analysis techniques (Hanedar et al. 2014). Similarly, for PMF a critical step is determining the selection of correct number of modeling parameters otherwise it makes the results challengeable (Callén et al. 2013). Summary of various source apportionment techniques used for PAHs among different environmental media in SAR was presented in Table 3.

Table 3 Summary of the source apportionment techniques used for PAHs

Matrix	SAR region	Rest of the world	Source Apportionment Techniques	Identified possible sources	References
Atmospheric	India		DMR's	coal and biofuel combustion	Rajput and Lakhani (2010)
	India		PCA	Biomass combustion	Masih et al. (2012)
	India		PCA	diesel and natural gas combustion	Likhani (2011)
	India		PCA	Diesel and gasoline vehicular combustion	Sharma et al. (2007)
	India		PCA	Diesel and gasoline vehicular combustion	Sharma et al. (2008)
	India		DMR's	Vehicular emissions	Sing et al. (2011)
	Pakistan		CMB	Biomass combustion	Stone et al. (2010)
	Nepal		DMR's	brick kiln combustion, vehicular emissions	Kishida et al. (2009)
		Spain	PMF	Stationary and vehicular emissions	Callén et al. (2013)
		Egypt	PCA-MLR, PMF, Unmix	Diesel and gasoline emissions	Khairy and Lohmann (2013)
		UK	PMF	traffic coal and wood burning	Jang et al. (2013)
		China	PMF, PCA, Unmix	biomass and fossil fuel combustion	Yang et al. (2013)
		U.S.	PMF	primary, mobile and combustion sources	Zang et al. (2009)
	Sri Lanka		PCA	Vehicular emissions, biomass combustion	Karunaratne and Wijayarathna (2013)
Water	India		DMR's	Petrogenic and Pyrogenic combustion	Ranjan et al. (2012)
	Nepal		DMR's	Pyrogenic combustion	Guzzella et al. (2011)
	Srilanka		DMR's	Vehicular emissions	Pathiratne et al. (2007)
	Pakistan		DMR's, PCA	Industrial activities, coal and trash burning	Farooq et al. (2011)
	Pakistan		DMR's, PCA	domestic and industrial waste water Discharge, vehicular emissions	Aziz et al. (2014)

(continued)

Table 3 (continued)

Matrix	SAR region	Rest of the world	Source Apportionment Techniques	Identified possible sources	References
Soil/Dust/ Sediment	Pakistan		DMR's	Land use petroleum activity	Din et al. (2013)
	Pakistan		DMR's, PCA	Brick kiln combustion	Kamal et al. (2015)
	India		DMR's, PCA	biomass and fossil fuel combustion	Agarwal et al. (2009)
	India		DMR's, PCA	Aircraft exhaust	Ray et al. (2011)
	Nepal		PCA	Traffic and industrial emissions	Aichner et al. (2011)

5 Ecological Risk Assessment

In an ecological system, risk quotients (RQ) have been used to evaluate the risk posed by environmental pollutants. For the estimation of ecosystem risk associated with PAHs contamination in water bodies, sediments and soils of South Asia, we evaluated the risk quotients of negligible concentration $RQ_{(NCs)}$ and the maximum permissible concentration $RQ_{(MPCs)}$ for ∑PAHs. Due to unavailability of negligible concentration (NCs) and maximum permissible concentrations (MPCs) set in the South Asian countries, we have used values reported by Kalf et al. (1997), for RQ evaluation. The methodology used for the risk evaluation is also described previously (Cao et al. 2010) and the classification of risk for ∑PAHs is presented in Table 4. $RQ_{\sum PAHs}$ (NCs) >1 for studies reported in water, sediments and soil of South Asia indicating that PAHs are of concern in this region. By comparing RQs of water for ∑PAHs with the classification system, it can be seen that the risks associated with ∑PAHs in waters of Bangladesh and India were calculated high. While for studies reported for aquatic system in Pakistan and Sri Lanka revealed moderate risk, and of lower level risks for Sagarmatha Park in Nepal.

According to the classification system established for the determination of PAHs levels, if the concentration of PAHs is greater than 1000 ng g^{-1} then the pollution levels for soil and sediment are considered as high (Baumard et al. 1998). The concentration of ∑PAHs in soil and sediments of South Asia region was greater than 1000 ng g^{-1} indicating high pollution level in the region. The resulting $RQ_{\sum PAHs}$ (NCs) and $RQ_{\sum PAHs}$ (MPCs) values of soil and sediment were high in South Asia.

Table 4 Risk level of PAHs in South Asia

	∑RQ (NCs)	∑RQ (MPCs)	Risk Level	Location	References
Water	749,881.0	7498.8	High	Bangsai River	Hossain et al. (2011)
	136,422.3	1364.2	High	Buriganga	Motaaleb et al. (2003)
	31,611.8	310.2	High	Gomti River	Malik et al. (2004)
	420.8	3.2	Moderate risk 2	Mumbai Harbor	Pandit et al. (2006)
	2706.7	27.1	High	Yamuna River	Shabeer et al. (2013)
	103,056.1	1030.2	High	Harbor line Mumbai	Dhananjayan et al. (2012)
	16,008.9	155.8	High	Gomti River	Malik et al. (2011)
	471.8	1.3	Moderate risk 2	Chenab River	Farooq et al. (2011)
	607.9	4.7	Moderate risk 2	Rawalpindi	Saba et al. (2012)
	155.5	1.5	Moderate risk 2	Beira lake	Pathiratne et al. (2007)
	300	0.88	Low risk	Sagarmatha National Park	Guzzella et al. (2011)
	133.2	1.3	Moderate risk 2	Bolgoda lake	Pathiratne et al. (2007)
Soil	14,352.0	143.5	High	Islamabad	Din et al. (2013)
	15,050.6	149.7	High	Rawalpindi	
	952,280.5	952,280.5	High	Delhi	Agarwal et al. (2009)
	3576.1	33.7	High	Agra, Industrial	Masih and Taneja 2006
	4080.6	39.1	High	Agra, Road side	
	2246.4	19.0	High	Agra, Residential	
	2870.7	21.7	High	Agra, Agriculture	
	1515.2	12.5	High	Delhi airport	Ray et al. (2008)
Sediment	535.6	2.6	High	Sewri	Dhananjayan et al. (2012)
	3670.8	34.3	High	Mahul	
	1154.9	8.3	High	Nhava	
	299.0	2.1	Moderate risk 2	Gomti River, India	Malik et al. (2011)
	5664.1	54.0	High	Yamuna River, India	Agarwal et al. (2006)

RQ-PAHs (NCs) = Low (>1; <800); moderate (>800); or <800; Higher (>800); RQ-PAHs (MPCs) = Risk free (0); Low (0); moderate (0–1); High (>1) (Cao et al. 2010)

6 Conclusions

This review summarizes the studies reported from South Asia for PAHs contamination of water, air, soil/sediment and biota. In South Asia, there is a lack of scientific research and monitoring studies to an extent that gives the clear picture of pollution in the region. Therefore detailed studies are required to assess the occurrence, sources and distribution of PAHs in this region as well as to examine the carcinogenic and mutagenic potential of these pollutants. Collective efforts or collaborations should be made even among the countries to maintain a proper database to pursue further research along with awareness. Monitoring studies indicated high concentrations of PAHs in the South Asian region. Anthropogenic activities have increased the levels of these pollutants not only in South Asian region but on a global scale. General trend of PAHs contamination in soil/sediments were followed as India > Sri Lanka > Pakistan and for water it's Bangladesh > India > Pakistan > Sri Lanka. Risk assessment conducted in this review also revealed the fact that South Asia is highly polluted in term of PAHs contamination. It is therefore highly recommended that proper measures should be taken to avoid PAHs contamination. Therefore, it is urgent to establish a monitoring program for PAHs, to ensure that any excess in concentrations over environmental quality standards is rapidly reported and necessary mitigation actions are taken.

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Conflict of interest
The authors declare no competing financial interest.

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Environmental Pollution, Toxicity Profile and Treatment Approaches for Tannery Wastewater and Its Chemical Pollutants

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1 Introduction

Leather industries (LIs) play an important role in the national economy of many developing countries like India, China, Turkey, Brazil, Ethiopia, Pakistan and Bangladesh (Leta et al. 2004; Lefebvre et al. 2006; Kurt et al. 2007; Verma et al. 2008; Haydar and Aziz 2009; Lofrano et al. 2013; Chowdhury et al. 2013; Wang et al. 2014). Approximately, 22,700.5 M ft^2 (or 2108.94 M mt^2) of leather is produced annually in the world (FAO 2008) and the world trade for the leather sector is estimated as US$100 billion per year (UNIDO 2010). The demand for leather and leather products is ever increasing and independent of supply. The United States, Germany and other European countries are the major importers whereas the countries like India, China, Pakistan, Egypt, Brazil, Thailand and Indonesia are the major exporters of leather and leather products.

Unfortunately, LIs are also one of the major polluters worldwide because of the complex nature of their wastewaters. During leather production, a variety of chemicals with large volumes of water are used to convert the raw hide/skins into leather or leather products generating large volumes of high strength wastewater, which are a major source of environmental pollution. The wastewater generated is characterized by a high chemical oxygen demand (COD), biological oxygen demand (BOD), Total dissolved solids (TDS), Total suspended solids (TSS), chromium (III) and phenolics with high pH, strong odor and dark brown color (Durai and Rajasimmam 2011; Suganthi et al. 2013; Dixit et al. 2015). Apart from high organic content, tannery wastewater (TWW) also contains various nutrients such as nitrogen and phosphorus that can lead to eutrophication of water bodies (Rai et al. 2005; Durai and Rajasimmam 2011; Raj et al. 2014). In addition, the dark brown color of wastewater hinders the photosynthesis process by blocking the sunlight penetration and it is therefore deleterious to aquatic life (Aravindhan et al. 2004; Rai et al. 2005; Kongjao et al. 2008; Mwinyihija 2010; Durai and Rajasimmam 2011). However, the major pollutants present in TWW include chromium, tannins or syntans (STs), phenolics, phthalates and azo dyes (Kumar et al. 2008; Lofrano et al. 2013; Dixit et al. 2015).

The high concentration and low biodegradability of pollutants present in TWW is a major cause of serious environmental concern (Di Iaconi et al. 2002; Schrank et al. 2009) and thus, it is imperative to adequately treat the TWW before its final disposal in the environment. However, the increasingly stringent environmental regulations are also forcing the LIs to improve the treatment processes applied at wastewater treatment plants (WWTPs) and also explore the alternative methods for the better treatment and management of TWW.

Therefore, this paper highlights the environmental impacts and toxicity profile of TWW and chemicals and provides a detailed review on the existing treatment approaches for its safe disposal into the environment. The emerging treatment approaches have been discussed with their merits and demerits. Further, the emerging anammox technology for the removal of ammonia from TWW and constructed wetlands (CWs) for wastewater treatment has been discussed. In addition, the clean

technologies (CTs) for waste minimization, control and management in LIs are discussed. Moreover, the international legislation scenario on discharge limits for TWW and chemicals has also been discussed country wise with discharge standards to prevent the environmental pollution.

2 Leather Production and Chemicals Used in Tanning Process

LIs are specialized in processing of hide (skins of large animals like cows, buffaloes and horses) and skins (skins of small animals like sheep, goats and calves) for leather production. The tanning process used to convert the hide/skins (a highly putrescible material) into stable and imputrescible products termed as leather, which is used for various purposes (Dixit et al. 2015). Tanning processes are classified into vegetable or chrome tanning depending on the type of tanning reagent (tannins or chromium) applied (Ram et al. 1999; Mannucci et al. 2010) (Table 1). The steps and overall process of leather production are well described in

Table 1 Comparison between vegetable tanning and chrome tanning process

S. No.	Parameters	Vegetable tanning	Chrome tanning
1.	Tanning agent	Vegetable tannins (VTs)	Chromium salt
2.	Nature	Organic tanning	Inorganic (mineral) tanning
3.	Action	Slow process	Fast process
4.	Cost	Costly affairs	Cost effective
5.	Time	Time consuming	Less time consuming
6.	Geographical use	Used in developed countries and few developing countries	Used in developing countries
7.	Products	Heavy leather like shoe soles, luggage, saddlery and belt etc.	Light weight leathers like shoe uppers, garments and bag etc.
8.	Product characteristics	Higher thermal stability and water resistant	Softer and more pliable leather
9.	Processing steps	All the steps are same as in chrome tanning process	Additionally, retanning, dyeing and fatliquoring are usually performed to produce finished leather and a preliminary degreasing step may be necessary when using animal skins, such as ship skins
10.	Environmental Impact	Does not require prior preparation of pickling and therefore contribution to pollution load from sulfate salts are lower hence ecofriendly, but VTs are hard to biodegrade. Thus, waste bearing VTs degrade slowly	Generation of chromium containing sludge and wastewater is still a major environmental problem of chrome tanning process

the literature (Thanikaivelan et al. 2005; ILTIP 2010; Lofrano et al. 2013; Dixit et al. 2015). However, the tanning process involves different steps and chemicals for different end products and the kind and amount of waste generated may vary in a wide range of quantity and nature (Lofrano et al. 2013).

During the tanning process, a large amount of chemicals such as acids, alkalis, chromium salts, tannins, sulfates, phenolics, surfactants, dyes, auxiliaries, sulphonated oils and biocide etc. are used to convert the semi-soluble protein "collagen" present in hide/skins into highly durable commercial forms of leather, and the chemicals used are not completely fixed by the hide/skins and end up in wastewater (Lofrano et al. 2008; Mannucci et al. 2010). The poor uptake of chromium salt (50–70 %) during the tanning process results in the material wastage on one hand and disturbance of the ecological balance on the other hand (Saravanbahavan et al. 2004; Dixit et al. 2015). Moreover, the sulfonated oils and synthetic tannins or syntans (STs) (an extended set of chemicals such as phenol, naphthalene, formaldehyde, melamine and acrylic resins) are also used in tanning/retanning process to make the leather more softer (Lofrano et al. 2008, 2013).

Many regulations have been passed to avoid the use of hazardous chemicals in industrial processes such as Integrated Pollution Prevention and Control Directive (96/61/EC 1996; 2008/1/EC 2008). The Directive (REACH) (EC 1907/2006) for European Regulatory Framework on chemicals namely Registration, Evaluation, Authorization and Restriction of Chemical substances directed the LIs to avoid the use of those leather auxiliaries and basic chemicals, which are not registered and listed in the Safety Data Sheet (Lofrano et al. 2013). Moreover, the Directive (2003/53/EC) restricted the marketing and use of products/product formulations containing >0.1 % of nonyl ethoxyphenol (NPE) or nonylphenol (NP) and their use in making of the leather products in Europe (Lofrano et al. 2008). In addition, the Directive (1999/815/EC) has directed the industries to label the products if they contain >0.5 % phthalates (benzyl butyl phthalate, di-butyl phthalate and di-ethyl hexyl phthalate) due to the reproductive toxic potential of the phthalates (EU 2003). The use of o-phenyl phenol is restricted for leather finishing due to its carcinogenic potential (EPA 2007) and the use of formaldehyde (a cross liker casein top coats) due to its carcinogenic potential has been also restricted (EU 1998). The inorganic compounds such as cadmium sulfate and lead chromate (fastening agents) are highly toxic in nature (IARC 2004; ATSDR 2008). Further, the EU Azo Colorants Directive (2002) has prioritized several azo dyes and restricted their use in LIs due to higher toxicity but there is no any particular restriction to use STs yet in LIs worldwide (Dixit et al. 2015).

3 Tannery Wastewater: Nature and Characteristics

Water is crucial for life and also used in many industrial processes. In the tanning process, a large quantity of water and chemicals are used to treat raw hide/skins and approximately 30–35 m^3 of wastewater is generated per ton of raw hide/skins processed (Lofrano et al. 2008; Islam et al. 2014). However, the wastewater

generation depends on the nature of raw material, finishing product and production processes applied (Tunay et al. 1995; Lofrano et al. 2013). This presents two major problems for LIs: First, the availability of good quality of water and second is the adequate treatment of such a large volume of highly contaminated wastewater.

Tannery wastewater (TWW) is a basic, dark brown coloured waste having COD, BOD, TDS, chromium (III) and phenolics with high pH and strong odor (Durai and Rajasimmam 2011; Suganthi et al. 2013; Dixit et al. 2015). However, the characteristics of TWW may vary from industry to industry, raw materials and chemicals used, type of final product and the production processes adopted by LIs (Apaydin et al. 2009; Rameshraja and Suresh 2011; Lofrano et al. 2013).

During leather production, the beamhouse and tanning operation are the high pollution causing steps because beamhouse operation contributes high organic and sulfide content whereas tanning operation contributes high salts (of chloride, ammonium, chromium and sulfate) concentrations in TWW (Cooman et al. 2003; Rameshraja and Suresh 2011). Hence, the beamhouse wastewater is characterized by an alkaline pH and tanning wastewater by a very acidic pH as well as a high COD value (Lofrano et al. 2013). Generally, TWW is highly rich in nitrogen, especially organic nitrogen, but very poor in phosphorous (Durai and Rajasimmam 2011). The retanning streams relatively have a low BOD and TSS (Total suspended solids), but high COD and contain trivalent chromium (III), tannins, sulfonated oils and spent dyes whereas the wet finishing, retanning, dyeing and fat liquoring processes contribute low fractions of salt in TWW that is predominantly originating from the hide/skins in the soak liquor (USEPA 1986; Lofrano et al. 2013). Further, BOD_5/COD (due to inhibitors) or BOD_5/TOC (due to high sulfide and chloride concentration) ratio is used for the biodegradation study of TWW (Lofrano et al. 2013). The data on wastewater generation and pollution load of each step during the processing of raw hide/skins are presented in Table 2.

Table 2 Pollution load and quantity of wastewater generated during the processing of per ton raw hide/skins

	Processing operation (load kg/ton of raw hide/skins)					
Pollution load	Soaking	Unhairing/ liming	Deliming and bating	Chrome tanning	Post-tanning	Finishing
Wastewater generated (m^3 or kL)	9.0–12.0	4.0–6.0	1.5–2.0	1.0–2.0	1.0–1.5	1.0–2.0
TSS	11–17	53–97	8–12	5–10	6–11	0–2
COD	22–33	79–122	13–20	7–11	24–40	0–5
BOD	7–11	28–45	5–9	2–4	8–15	0–2
Cr	–	–	–	2–5	1–2	–
Sulphides	–	3.9–8.7	0.1–0.3	–	–	–
NH_3-N	0.1–0.2	0.4–0.5	2.6–3.9	0.6–0.9	0.3–0.5	–
TKN	1–2	6–8	3–5	0.6–0.9	1–2	–
Chlorides	85–113	5–15	2–4	40–60	5–10	–
Sulfates	1–2	1–2	10–26	30–55	10–25	–

Adapted from Dixit et al. (2015)

4 Environmental Pollution and Toxicity Profile of Tannery Wastewater

TWW is ranked as one of the major environmental pollutants among all the industrial wastewaters (Verma et al. 2008; Gupta et al. 2012). The presence of a variety of toxic and hazardous chemicals such as chromium, chlorophenols, formaldehydes, STs, oils, resins, biocides, detergents and phthalates etc. in TWW creates a negative image of LIs (Lofrano et al. 2013; Dixit et al. 2015). The toxicity of chemicals used during leather processing is summarized in Table 3. The wastewater generated from Common Effluent Treatment Plant (CETP) contains high BOD, COD, TDS and a variety of toxic heavy metals especially chromium, which makes it potentially toxic for humans and other living beings (Mondal et al. 2012; Lofrano et al. 2013; Dixit et al. 2015). In addition, TWW also contains a mixture of chemical compounds, which are used during leather processing and are not get properly degraded even after the conventional treatment and have a negative impact on living organisms and environment (Alvarez-Bernal et al. 2006; Oral et al. 2007; Kumar et al. 2008; Tigini et al. 2011; Siqueira et al. 2011; Shakir et al. 2012; Lofrano et al. 2013; Saxena and Bharagava 2015).

Table 3 Applications, toxicity and LD_{50} for chemicals used during leather production in leather industry (Adapted from Kumar et al. 2008; Dixit et al. 2015)

Name of chemicals	Applications	LD_{50} in rats (oral mg/kg)	Target organs
Pentachlorophenol (PCP) (a carcinogen)	Applied as a biocide in preservative for raw hides/skins	2000	Eyes, nose, skin, respiratory tract, blood, kidney, liver, immune system and reproductive system
Di-butyl phthalate (DBP) (a endocrine disrupting chemical)	Applied as a plasticizer in artificial leather manufacturing	7499	Eyes, lungs, gastrointestinal (GI) tract and testes
Benzyl butyl phthalate (BBP) (a endocrine disrupting chemical)	Applied in preparation of micro-porous artificial leather coating/water vapour-permeable sheet materials	2330	Eyes, lungs, liver and reproductive system
Bis(2-ethylhexyl) phthalate (DEHP) (a endocrine disrupting chemical)	Applied as a plasticizer in artificial leather manufacturing	30,000	Liver and testes
Short chain, chlorinated paraffin's	Additive for leather treatment (gives smoothness), leather clothing and belts and as oiling agent	3090	Liver, kidney and thyroid

(continued)

Table 3 (continued)

Name of chemicals	Applications	LD_{50} in rats (oral mg/kg)	Target organs
Anthracene (a carcinogen)	Additive during tanning	16,000	Kidneys and liver
Nonyl phenol (a endocrine disrupting chemical and xenoestrogen)	Applied during finishing	1475	Blood. Lungs, eyes, skin, central nervous system (CNS), kidneys and testes
N-methyl pyrrolidone	Applied as a coalescene, plasticizers and wetting agents	3914	Eyes, kidneys, lymphatic system, liver, lung and testes
Methyl isothiazolinone (a carcinogen)	Applied as biocide	1800	Skin and eyes
Organotin compounds (Dibutyl tin) (a carcinogen)	Applied as a catalyst	175	GI tract and liver
Azo dyes (Orange II) (a carcinogen)	Applied as a dyeing agent	3418	Blood, liver and testes
Hexachlorobenzene (a carcinogen)	Applied for raw hide/skins preservation	10,000	Reproductive system
Chromium (a carcinogen)	Applied as a tanning agent	3250	Kidneys, CNS and hematopoietic system
Formaldehyde (a carcinogen)	Applied in finishing of leather	100	Eyes and lungs
Arsenic (a carcinogen)	Applied in finishing of leather	763	Liver, kidneys, skin, lungs and lymphatic system
Sodium dichromate	Applied in preparation of chrome-tanning salts	NA	Blood, kidneys, heart, lungs and eyes
Cobalt dichloride	Applied in dyeing and finishing	80	Skin, lungs, liver, kidney and heart
Cadmium sulfate (Pigment)	Applied as fastening agents and used in marking and surfacing of material.	280	Lungs, liver, tissues and reproductive system
Lead chromate (pigment)	Applied as fastening agents and used in marking and surfacing of material.	1000	Lungs, liver, tissues and reproductive system

NA not available

TWW is a major source of water and soil pollution. The dark brown color blocks the sunlight penetration, and thus, reduces the photosynthetic activity and oxygenation of receiving water bodies and hence, becomes detrimental to aquatic life (Song et al. 2000; Kongjao et al. 2008; Bakare et al. 2009; Mwinyihija 2010; Carpenter et al. 2013). In addition, the depletion in dissolved oxygen encourages the anaerobic condition, which leads to the putrefying odour of receiving water bodies (Rai et al. 2005; Sahu et al. 2007; Verma et al. 2008). TWW also causes

eutrophication of polluted water bodies and thus adversely affecting the ecological functioning of aquatic resources (Rai et al. 2005; Durai and Rajasimmam 2011; Schilling et al. 2012; Dixit et al. 2015). The high concentration of heavy metals in sediments of the Ganga river and its tributaries has been reported (Singh et al. 2003; Tare et al. 2003; Bhatnagar et al. 2013). The increase in the salinisation of rivers and groundwater has led to the reduction in soil fertility and quality of drinking water in Tamil Nadu, India (Money 2008). It has been estimated that over 55,000 ha of land has been contaminated by TWW and around five million peoples are affected by low quality of drinking water and social environment (CSIRO 2001; Sahasranaman and Jackson 2005). TWW is also reported to inhibit the nitrification process (Szpyrkowicz et al. 2001; Trujillo-Tapia et al. 2008; Lofrano et al. 2013) as well as to cause a huge foaming problem on surface waters (Schilling et al. 2012).

Moreover, the treated/partially treated TWW causes severe toxic effects in fishes and other aquatic organisms. The genotoxicity and mutagenicity of water polluted with TWW has been evaluated by the micronucleus test and the comet assay by using fish *Oreochromis niloticus* (Matsumoto et al. 2006). De Nicola et al. (2007) have studied the toxicity of mimosa tannin and phenol-based syntans on sea urchin (*Paracentrotus lividus* and *Sphaerechinus granularis*) during the early developmental stages and on marine algal cell growth (*Dunaliella tertiolecta*) and reported the sea urchin embryogenesis was affected by vegetable tannins and syntan water extracts at a level of 1 mg L^{-1}. Afaq and Rana (2009) also studied the impact of leather dyes (Bismarck brown and acid leather brown) on the protein metabolism in fresh water teleost, *Cirrhinus mrigala* (Ham.) and reported a significant decrease in total protein content in teleost treated with leather dyes. In addition, the toxic effects of TWW on the survival and histopathological parameters in the different organs of fishes *Channa punctatus* and *Oreochromis mossambicus* have been studied (Mohanta et al. 2010; Navaraj and Yasmin 2012). However, the toxic effects of TWW on the hematological parameters of a common fish *Tilapia mossambica* and fresh water fish, *Labeo rohita* (Hamilton) has also been recently studied (Lesley Sounderraj et al. 2012; Praveena et al. 2013). Further, TWW was also reported to interfere with the metabolic processes by altering the activity of oxidative enzymes in different organs of guppy fish, *Poecilia reticulate* and thereby causing cellular injury as a result of exposure (Aich et al. 2011, 2015).

Further, the presence of pathogens in water and wastewater has been reviewed by many workers (Bharagava et al. 2014; Saxena et al. 2015). TWW are also highly rich in organic and inorganic constituents and thus, may provide a chance to a variety of pathogenic bacteria to flourish and contaminate the receiving water bodies as these constituents may act as a source of nutrients (Verma et al. 2008; Bharagava et al. 2014). Recently, Chandra et al. (2011) have reported the presence of various types of organic pollutants (OPs) and bacterial communities in two aeration lagoons of a CETP used for the degradation and detoxification of TWW in India and also tested the toxicity of TWW on mung bean (*Phaseolus mungo*) in terms of seed germination and seedling growth. In addition, various authors have also assessed the bacteriological quality of TWW and reported the presence of a variety of pathogenic bacteria remained in TWW even after the secondary treatment process (Verma et al. 2008; Ramteke et al. 2010; Bharagava et al. 2014).

Generally, LIs discharges their wastewater into nearby canals/rivers, which are directly/indirectly being used by farmers for the irrigation of agricultural crops (Trujillo-Tapia et al. 2008; Gupta et al. 2012). This practice leads to the movement of potentially toxic metals like chromium from water to crop plants that ultimately reach into the human/animal body and cause toxicity (Sinha et al. 2008; Chandra et al. 2009). However, the chromium toxicity mainly depends on the chemical speciation and thus, the associated health effects are influenced by the chemical forms of exposure (Rameshraja and Suresh 2011). It is well reported that chromium (VI) is a potent carcinogen for humans, animals, plants as well as microbes as it enters the cells *via* surface transport system and get reduced into chromium (III) form and causes various genotoxic effects (Ackerley et al. 2004; Aravindhan et al. 2004; Matsumoto et al. 2006; Tripathi et al. 2011; Raj et al. 2014). Thus, the use of Cr loaded TWW for the irrigation of agricultural crops disrupts the several physiological and cytological processes in cells (Shanker et al. 2005; Chidambaram et al. 2009; Gupta et al. 2012) leading to the reduction in root and shoot growth and biomass, seed germination, seedling growth (Lopez-Luna et al. 2009; Hussain et al. 2010), and also induces the chlorosis, photosynthetic impairment and finally leading to the plant death (Akinici and Akinci 2010; Asfaw et al. 2012). However, the effect of TWW on seed germination and seedling growth is governed by its concentration and it is crop-specific. In a recent study conducted on mung bean (*Vigna radiate* (L.) wilczek) by Raj et al. (2014), the percent inhibition of seed germination was 90 % and 75 %, when seeds were treated with 25 % untreated and treated TWW, respectively. Moreover, it is also reported that treated and adequately diluted TWW can be used for the irrigation of agricultural crops as it provides a reliable source of water supply to farmers and contains valuable plant nutrients especially N, P, K and also add organic matter to soil (Trujillo-Tapia et al. 2008; Durai and Rajasimmam 2011; Asfaw et al. 2012; Sangeetha et al. 2012; Kohli and Malaviya 2013). Further, the genotoxic and mutagenic effects of TWW and agricultural soil irrigated with TWW has been recently studied (Alam et al. 2009, 2010).

In addition, the inappropriate discharge of TWW also leads to significant levels of soil pollution as well as acidification because of high salt loads in wastewater (Chowdhury et al. 2004; Alvarez-Bernal et al. 2006; Mwinyihija 2010; Raj et al. 2014). High sulfide content in TWW also causes the deficiency of some micronutrients in soil such as Zn, Cu and Fe etc. (Raj et al. 2014). However, Cr (VI) alters the structure of soil microbial communities and reduces their growth and finally retards the bioremediation process and if it enters into the food chain, causes skin irritation, eardrum perforation, nasal irritation, ulceration and lung carcinoma in humans as well as animals along with accumulation in placenta impairing the fetal development in mammals (Cheung and Gu 2007; Chandra et al. 2011; Asfaw et al. 2012). In addition, the exposure to chlorinated phenols is possible particularly to pentachlorophenol (PCP), which is highly carcinogenic, teratogenic and mutagenic in nature and causes toxicity to living beings by inhibiting the oxidative phosphorylation, inactivating the respiratory enzymes and damaging the mitochondrial structure (Jain et al. 2005; Verma and Maurya 2013; Tripathi et al. 2011).

The high concentration of PCP can also cause the obstruction in circulatory system of lungs, heart failure and damage to central nervous system (USDHHS 2001; Tewari et al. 2011; Dixit et al. 2015).

In addition, TWW also contain azo dyes that are highly persistent in nature due to their complex chemical structure and xenobiotic nature leading to the environmental pollution (Nachiyar and Rajkumar 2003; Gurulakshmi et al. 2008; Mahmood et al. 2013; Baccar et al. 2011; Patel et al. 2012; Preethi et al. 2013; Dixit et al. 2015). Thus, the removal of azo dyes from TWW is essential because of their high mutagenicity, carcinogenicity and intense coloration problems of contaminated aquatic resources (Osugi et al. 2009; Saratale et al. 2010). The discharge of azo dyes into the surface water also leads to the aesthetic problems and obstruct the light penetration and oxygen transport into the water bodies and finally affecting the aquatic life (Khalid et al. 2008; Chen et al. 2011). Moreover, these dyestuffs have been also reported to cause some other serious problems such as dermatitis, skin and eye irritation and respiratory problems in human beings (Keharia and Madamwar 2003).

Further, there has been an increasing concern regarding the release of many endocrine disrupting compounds (EDCs) along with TWW in environment. EDCs disturb the delicate hormonal balance and compromise the reproductive fitness of living beings and ultimately may lead to carcinogenesis (Dixit et al. 2015). Kumar et al. (2008) have detected many EDCs like nonylphenol (NP), 4-aminobiphenyl, hexachlorobenzene and benzidine in TWW collected from the northern region of India and tested their toxicity on the reproductive system of male rats. However, the presence of phthalates (EDCs) such as bis(2-ethylhexyl)phthalate (DEHP), dibutyl phthalate (DBP), bis(2-methoxyethyl)phthalate in TWW has been also reported (Alam et al. 2009, 2010). Therefore, the adequate treatment of TWW prior to its final disposal into the environment is required.

5 Treatment Approaches for Tannery Wastewater and Chemicals

TWW is a major source of soil and water pollution and it is therefore essential to adequately treat the TWW prior to its safe disposal into the environment. This can be achieved by using physical, chemical and biological methods either alone or in combination.

5.1 Physico-Chemical Treatment Approaches

5.1.1 Coagulation/Flocculation

Coagulation is the destabilization of colloids by neutralizing the forces that keep them apart. Cationic coagulants provide positive charge to reduce the negative

charge (zeta potential) of the colloids. As a result, the particles collide to form larger particles (flocs) whereas flocculation is the action of polymers to form bridges between the flocs, and bind the particles to form large agglomerates or clumps. There are a number of coagulants such as aluminium sulfate ($AlSO_4$), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$) etc. that are used to reduce the organic load (COD) and suspended solids (SS) as well as to remove toxic metals mainly chromium from TWW (Lofrano et al. 2013).

However, coagulants are pH specific and their effectiveness largely depends on their type and concentration and characteristics of the wastewater to be treated (Song et al. 2004). Ates et al. (1997) reported >70 % removal of COD and <5 mg L^{-1} of total chromium from TWW using alum and $FeCl_3$ based-CF. Song et al. (2004) also reported 30–37 % removal of total COD, 74–99 % of chromium and 38–46 % of SS by using 800 mg L^{-1} of alum at pH 7.5 from TWW containing 260 mg L^{-1} of suspended solids, 16.8 mg L^{-1} of chromium, 3300 mg L^{-1} of COD at pH 9.2 and finally concluded that $FeCl_3$ based CF proved better results than alum based-CF. Chowdhury et al. (2013) have reported 92 % removal of COD and 96 % of chromium from TWW using $FeCl_3$ at the concentration of 150 mg L^{-1} at pH 7 followed by sand-stone filtration process. In addition, Shegani (2014) also reported 81.60 %, 98.34 %, 92 %, 75.00 %, 70.00 %, 69.20 % and 50 % removal of COD, ammonia, nitrate, hexavalent chromium, phosphate, chloride and H_2S, respectively by using coagulants $Ca(OH)_2$ and $FeSO_4 \cdot 7H_2O$, but a low reduction in sulfate (19.00 %) and TSS (13.00 %) and an increase in TDS (15.60 %) were observed.

Moreover, some coagulants such as poly-aluminium chloride (PAC), poly-aluminium silicate (PASiC) and poly-aluminium ferric chloride (PAFC) ($[Al_2(OH)nCl_6\text{-}n]m.[Fe_2(OH)nCl_6\text{-}n]m$) have been developed with improved coagulation efficiency to minimize the residual coagulants in treated wastewater (Gao et al. 2004; Lofrano et al. 2013). Lofrano et al. (2006) reported >75 % removal of COD and >95 % of TSS from TWW at all doses of alum (800–900–1000–1200 mg L^{-1}) using PAFC (900 mg L^{-1}) at pH 8.5. Yoganand and Umapathy (in press) have also applied a green methodology for the recovery of chromium (VI) from TWW using newly synthesized quaternary ammonium salt and reported 99.99 % removal of chromium (VI) from TWW.

5.1.2 Adsorption

Adsorption is typically used for the removal of toxic metals especially chromium from TWW. There are a number of studies available on the use of adsorbents such as bentonite clay, cement kiln dust, activated carbon etc. for the treatment of TWW (Fadali et al. 2004; Fahim et al. 2006; Tahir and Naseem 2007). Further, the use of chitin-humic acid based hybrid and ground shrimp shells as adsorbent for the significant removal of Cr(III) from TWW has been reported (Santosa et al. 2008; Fabbricino et al. 2013). Moreover, the use of lime/bitten based coagulants and activated carbon as a post treatment of TWW is also suggested (Ayoub et al. 2011).

5.2 *Biological Treatment Approaches*

Biological approaches are the eco-friendly methods for the treatment of industrial wastewaters and involve the stabilization of waste by decomposing them into harmless inorganic solids either by aerobic or anaerobic processes. The most commonly used processes for the biological treatment of TWW are the Activated sludge process (ASP) and Upflow Anaerobic Sludge Blanket (UASB) process (Durai and Rajasimmam 2011).

5.2.1 Aerobic Treatment

In an aerobic treatment process, the waste decomposition rate is fast and also not characterized by unpleasant odours but a large amount of sludge is generated. There are several studies on the aerobic treatment of TWW using ASP as has been reported earlier by many workers (Jawahar et al. 1998; Eckenfelder 2002; Tare et al. 2003; Vidal et al. 2004; Hayder et al. 2007; Ramteke et al. 2010) and some of the important findings are summarized in Table 4.

TWW is highly saline in nature due to high load of salts, which are used for the preservation of raw hides/skins (Sundarapandiyan et al. 2010) and therefore, causes some serious problems in the biological treatment of TWW. The major problems include (Sivaprakasam et al. 2008): (a) limited adaptation of conventional cultures due to higher salt concentration (>3–5 % w/v), that therefore could not effectively treat TWW (b) salt adaptation of cultures is easily lost when subjected to salt free medium, and (c) changes in the ionic strength (salt concentration from 0.5 to 2 % w/v) cause cell disruption even with the acclimatized cultures and finally lead to system failure.

However, the high concentration of poorly biodegradable compounds such as tannins and other toxic metals inhibit the biological treatment processes (Schrank et al. 2004). Cr (VI) is reported to inhibit the growth of heterotrophs as well as nitrifying/denitrifying bacteria (Stasinakis et al. 2002; Farabegoli et al. 2004). To overcome this problem, a Sequencing Batch Reactor (SBR) is highly efficient to carry out the biological treatment and nitrogen removal from TWW in the presence of inhibitors due to its low cost, flexible operation and selection and enrichment of a particular microbial species (Farabegoli et al. 2004; Ganesh et al. 2006; Murat et al. 2006; Durai and Rajasimmam 2011; Rameshraja and Suresh 2011; Faouzi et al. 2013; Lofrano et al. 2013).

Moreover, the fluctuation in temperature range also has adverse effects on the nitrification process. The fluctuation in the temperature range significantly affects the removal of organic carbon and nitrogen from TWW whereas it has a minor influence on COD removal efficiency (4–5 %) that has been studied for a full-scale activated sludge process based treatment plant used for TWW (Gorgun et al. 2007). Further, the improvement in the performance of the nitrification process through increased aeration and total nitrogen removal efficiency (up to 60 %) at a temperature range between 21 and 35 °C during an intermittent aeration type of operation has been reported (Insel et al. 2009).

Table 4 Microorganisms reported in the degradation of tannery wastewater

References	Microorganisms	COD removal (%)	BOD removal (%)	Cr removal (%)
Kim et al. (2014)	*Brachymonas denitrificans*	98.3	–	88.5
Noorjahan (2014)	*E. coli*	90	90	63.8
	Bacillus sp.	95.4	95.4	73.5
Elmagd and Mahmoud (2014)	Mixed culture	98.3	98.4	98.3
Sharma and Malaviya (2013)	*Fusarium chlamydosporium* SPFS2-g	71.80	–	–
Yusuf et al. (2013)	*B. subtilis*	87.6	–	–
	P. fragi	85.2		
El-Bestawy et al. (2013)	*Providencia vermicola* W9B-11, *Escherichia coli* O7:K1 CE10, *Bacillus sp.* 58, *Bacillus amyloliquefaciens* T004, *Pseudomonas stutzeri* M15-10-3, *Bacillus sp.* PL47	79.16	94.14	93.66
Mandal et al. (2010)	*Thiobacillus ferrooxidans*	69	72	5
Nanda et al. (2010)	*Nostoc* sp.	37.8	48.6	–
Ramteke et al. (2010)	*E. coli*	98.46	90	–
	Vibrio sp.	87.5		
	Pseudomonas sp.	96.15		
Sivaprakasam et al. (2008)	*P. aeruginosa*, *B. flexus*, *E. homiense*, *S. aureus*	80	–	–
Vankar and Bajpai (2008)	*Trichoderma* sp.	–	–	97.93
Onyancha et al. (2008)	*S. condensate*	–	–	>75
	R. hieroglyphicum			
Srivastava et al. (2007)	*Acenetobacter* sp.	–	–	90
Rajasimman et al. (2007)	Mixed culture	46–85	65–93	–
Wang et al. (2007)	*A. Thiooxidans*	–	–	99.7
Srivastava and Thakur (2006)	*Aspergillus* sp.	–	–	–
	Hirsutella sp.			70
Lefebvre et al. (2005)	Halophiles	95	–	–
Thanigavel (2004)	Mixed culture	89.5	–	–
Shakoori et al. (2000)	Bacterial strain	–	–	87

5.2.2 Anaerobic Treatment

The use of anaerobic treatment processes to treat TWW is an interesting option as compared to aerobic treatment process because of low energy consumption and sludge production. However, its full scale application has several drawbacks (Mannucci et al. 2010): i) continuous production of sulfide (from sulfate reduction) in absence of alternative electron acceptors such as oxygen and nitrate; ii) high protein content affects the selection of biomass, slow down the kinetics of hydrolysis and also inhibit the sludge formation, and iii) requirement of an additional aerobic treatment to meet the high COD removal.

The sulfide mainly inhibits the methanogenesis process during the anaerobic treatment of TWW and this might be due to the direct toxicity of sulfide, substrate competition between the sulfate reducing bacteria and methanogenic bacteria and precipitation of trace elements (Midha and Dey 2008; Rameshraja and Suresh 2011; Mannucci et al. 2014). However, the mechanisms of sulfide toxicity are not well understood.

The anaerobic treatment of TWW is mainly performed by using either the anaerobic filters (AF) composed of both upflow anaerobic filters (UAF) and down-flow anaerobic filters (DAF) or Upflow Anaerobic Sludge Blanket (UASB) reactors (Lefebvre et al. 2006; Rajasimman et al. 2007; El-Sheikh et al. 2011; Dixit et al. 2015). Beside these, the use of expanded granular sludge bed (EGSB) and anaerobic baffled reactor (ABR) for the treatment of TWW is also suggested (Zupancic and Jemec 2010).

In addition, the anaerobic treatment of TWW is more favorable in tropical countries having higher temperatures such as India, Pakistan, China, and Brazil etc. as compared to European countries (Durai and Rajasimmam 2011; Mannucci et al. 2014). In these countries, the spread of new and large industrial areas to establish the LIs favor the development of centralized WWTPs. However, the application of anaerobic treatment processes at large scale makes it possible to balance the high operation and management costs with energy saving over the traditional aerobic treatment processes.

5.2.3 Constructed Wetlands and Treatment Ponds

Constructed wetlands (CWs) are man-engineered, eco-friendly systems designed to remove the pollutants from highly polluted industrial and municipal wastewaters. The use of CWs for the treatment of industrial wastewater has developed rapidly in current years and is now successfully employed to remove a diverse array of pollutants from wastewaters.

The proper functioning of a wetland system depends on the complex relationship between the plants, microorganisms, soil, wastewater characteristics and operational parameters (Aguilar et al. 2008). In this regard, several efforts have been made to select the suitable plant species capable to tolerate and remove the

pollutants from TWW (Mant et al. 2004; Calheiros et al. 2007, 2008, 2012), selecting the suitable supporting media/substrate for proper growth and development of wetland plants (Calheiros et al. 2008), as well as to study the bacterial community dynamics in CWs (Aguilar et al. 2008; Calheiros et al. 2009a, b). The plant roots and rhizomes are the major sites of microbial degradation/transformation of pollutants and subsequently the purification of wastewater because microbes form a biofilm on root surface and substrates (Stottmeister et al. 2003; Gagnon et al. 2007; Munch et al. 2007). However, the availability of nutrients or other environmental parameters affects the biofilm formation (Kierek-Pearson and Karatan 2005). Therefore, the detailed profiling of complex microbial populations is required to understand the proper functioning of CWs and phytoremediation processes (Chandra et al. 2015). Culture-dependent techniques are known to be insufficient to study the microbial community structure because numerous microorganisms are unculturable in lab conditions (Ward et al. 1990). Hence, molecular techniques such as random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR) and denaturation gradient gel electrophoresis (DGGE), is used for the study of microbial community structure, composition and diversity in CW system (Calheiros et al. 2009a, 2012).

Mant et al. (2004) have studied the phytoremediation potential of *Penisetum purpureum*, *Brachiaria decumbens* and *Phragmites australis* in CWs for the removal of chromium (ranging from 10 and 20 mg Cr dm^{-3}) from TWW. In addition, the potentials of *Canna indica*, *Typha latifolia*, *P. australis*, *Stenotaphrum secundatum* and *Iris pseudacorus* in CWs for the treatment of TWW under two different hydraulic loading rates at 3 and 6 cm/day has been studied and it was found that only *P. australis* and *T. latifolia* were able to establish successfully (Calheiros et al. 2007). Further, these authors also evaluated *Arundo donax* and *Sarcocornia fruticosa* in two series of horizontal subsurface flow CWs used to treat TWW received from a conventional biological treatment plant and reported the removal of COD (51 and 80 %) and BOD_5 (53 and 90 %) for COD inlet: 68–425 mg L^{-1} and for BOD_5 inlet: 16–220 mg L^{-1} (Calheiros et al. 2012). In addition, the use of TWW as a growth medium for *Arthrospira* (*Spirulina*) has been recently suggested (Dunn et al. 2013). However, the chromium salt can be retained in wetlands with non-specialized supporting media (Dotro et al. 2012).

On the other hand, the use of treatment ponds for the treatment of TWW can also be an effective approach. The effect of different environmental parameters like pH, temperature and dissolved oxygen on the efficiency of a pilot-scale advanced integrated wastewater treatment pond system (AIWTPSs) used to treat TWW has been reported by Tadesse et al. (2004). They also suggested a combination of advanced facultative pond (AFP), secondary facultative pond (SFP) and maturation pond (MP) in a series for the effective treatment of TWW. Recently, Kumar and Sahu (2013) have designed the anaerobic pond (AP) for the treatment of TWW in Egypt.

5.3 *Emerging Treatment Approaches*

The TWW discharged even after the conventional treatment process still contains many refractory and recalcitrant organic pollutants (ROPs) and thus, require further treatment for environmental safety. Therefore, in order to overcome this problem, the use of emerging treatment technologies is increasing in recent years.

5.3.1 Membrane Technologies

Membrane technologies (MTs) are used for the mechanical separation/purification of industrial wastewater with the help of permeable membranes. MTs operate without heating and therefore use less energy than conventional thermal separation processes such as distillation, sublimation or crystallization. The use of MTs in LIs is becoming popular in current years because of continually reducing cost and ever extending application possibilities.

The MTs offer many economic benefits to the LI, especially the recovery of chromium from TWW (Lawanda et al. 2009; Ranganathan and Kabadgi 2011) and are used for purification/reuse of wastewater and chemicals of deliming/bating liquor (Gallego-Molina et al. 2013), reduction of pollution load due to unhairing and degreasing (De Pinho 2009; Wang et al. 2011), removal of salts as well as in the biological treatment of TWW for its reuse (Lofrano et al. 2013). Several membrane-based technologies such as cross flow microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and supported liquid membranes (SLMs) can be used for the removal of pollutants from TWW (Lofrano et al. 2013; Dixit et al. 2015). However, the use of reverse osmosis (RO) with a plane membrane has been suggested as a post treatment for the removal of refractory compounds such as chlorides and sulfates, and resulted in the production of high quality of permeate that allowed the reuse of tannery wastewater within the production cycle and thus, reduced the groundwater consumption (De Gisi et al. 2009). The economical evaluation of membrane filtration technologies has been discussed in detail by Scholz and Lucas (2003). The successful integration of MTs in a conventional purification process for TWW streams has been recently reported by Stoller et al. (2013).

5.3.2 Membrane Bioreactors

A membrane bioreactor (MBR) is the combination of a membrane process like microfiltration or ultrafiltration with a suspended growth bioreactor, and is now widely used for municipal and industrial wastewater treatment. MBRs offer several advantages over the conventional activated sludge treatment process (CASTP) such as elimination of sludge from settling basins, independence of process performance from filamentous bulking or other phenomena that affect the sludge settleability

(Munz et al. 2008; Suganthi et al. 2013; Dixit et al. 2015). The presence of tannins in TWW reduces the kinetics of nitrification without large differences between the biomass selected with either the CASTP or the MBR used (Munz et al. 2009). However, the major drawbacks of membrane application are the significant fouling due to clogging, adsorption and formation of cake layer by pollutants like residual organics, dyes, and other impurities onto the membrane (Srinivasan et al. 2012; Stoller et al. 2013). However, the extensive work is in progress to reduce the bio-fouling problem in MBRs. Further, a hybrid membrane bioreactor (HMBR), which is the integration of various treatment technologies, may be a solution to overcome the bio-fouling problem of MBRs. More recently, the efficiency of HMBR (activated sludge process + electro-coagulation) for the effective removal of COD and color from TWW satisfying the discharge limits set by Tamil Nadu Pollution Control Board (TPCB) India has been evaluated (Suganthi et al. 2013).

5.3.3 Anammox Technology

The anammox technology is used for the anaerobic removal of ammonia from TWW and it is currently emerging because of its low cost and energy consuming nature (Anjali and Sabumon 2014). It involves the anoxic oxidation of ammonia with nitrite as a preferred electron acceptor and consumes 50 % less oxygen, 100 % less organic carbon and saves 90 % of operational costs in sludge disposal as compared to the conventional nitrification/denitrification processes (Anjali and Sabumon 2014). Therefore, industries, producing wastewaters having a high concentration of ammonia, are showing increased interest in the anammox process. However, the long start-up time and inhibitive nature in the presence of organic carbon and NH_4-N limits its field applications. Therefore, it is imperative to develop the mixed consortium capable of anammox in the presence of organic compounds. Further, the development of mixed microbial consortium consisting of ammonia oxidizing bacteria, anammox bacteria, and denitrifying bacteria is also expected to treat the wastewaters containing both ammonia and organic carbon.

5.3.4 Advanced Oxidation Processes

Advanced oxidation processes (AOPs) refers to the set of chemical treatment processes that use strong oxidizing agents (O_3, H_2O_2) and/or catalysts (Fe, Mn, TiO_2) and sometimes also use the high-energy radiation, e.g., UV light (Schrank et al. 2004; Naumczyk and Rusiniak 2005; Srinivasan et al. 2012; Dixit et al. 2015). AOPs are based on the production and utilization of hydroxyl radicals, which are strong oxidizing agents and quickly and non-selectively oxidize a broad range of recalcitrant organic pollutants such as benzoquinone, benzene, phenols, chlorophenols, dyes and formaldehyde in less time (Lofrano et al. 2013; Dixit et al. 2015). Generally, the AOPs are used to treat the secondary treated wastewater and therefore known as tertiary treatment (Audenaert et al. 2011). In this, most of the pollutants get converted into

stable inorganic compounds such as H_2O, CO_2 and salts, i.e. they undergo mineralization (Rameshraja and Suresh 2011). The treatment efficiency of AOPs is mostly evaluated in terms of COD removal however, TOC is a more suitable parameter to study the state of mineralization (Schrank et al. 2004, 2005; Costa et al. 2008; Monteiro Paschoal et al. 2009). There are various types of AOPs such as fenton oxidation, photo-oxidation, photo-fenton oxidation, ozonation, photocatalysis and electrochemical treatment processes that are applied to treat the TWW (Rameshraja and Suresh 2011; Lofrano et al. 2013; Dixit et al. 2015). The overall goal of AOPs used for TWW treatment is to reduce the pollution load and toxicity to such an extent that the treated TWW may be reintroduced into the receiving water bodies or reused during the process. The important findings of various AOPs applied to treat the TWW are presented in Table 5.

Table 5 Findings of some advanced oxidation processes (AOPs) applied for the treatment of tannery wastewater

References	AOPs	Wastewater type	Influent COD (mg L^{-1})	Operation parameters and reduction in pollutants
Modenes et al. (2012)	Photo-Fenton ($UV/Fe^{2+}/H_2O_2$)	Equalized tannery wastewater	11,878	COD removal (90 %), TSS removal (50 %), Fe^{2+} (0.4 g L^{-1}) and H_2O_2 (15 g L^{-1}), Irradiation time (540 min)
Houshyar et al. (2012)	Ozone	Pre-alkalized tannery wastewater	2177	COD removal (30–70 %), Time (120 min), Ozone flow rate (1–8 g/h)
Di Iaconi et al. (2010)	Ozone	Biologically treated tannery wastewater	2900	COD removal (97 %), TSS removal (96 %), TKN removal (91 %), Surfactants removal (98 %), Color removal (96 %)
Sundarapandiyan et al. (2010)	Electrochemical treatment	Synthetic tannery wastewater	10,715	COD removal (89 %), pH 3–9, Current density (0.006–0.024 A cm^{-2}), Time (120 min)
Preethi et al. (2009)	Ozone	Raw tannery wastewater	5000	COD removal (60 %), O_3 flow rate (2×10^{-3} m^3 min^{-1}), Time (20–120 min) and pH (4)
Espinoza-Quinones et al. (2009)	Electrochemical treatment	Equalized tannery wastewater	17,618	COD removal (51–56 %), TSS removal (30–70 %), Electric current flow rate (0–10 A at 0–30 V), Time (30–45 min)
Costa et al. (2008)	Electrochemical treatment	Equalized tannery wastewater	1005 (TOC)	Maximum phenol removal (83.9 %), Maximum TOC removal (40.5 %), Time (5 h of electrolysis)

(continued)

Table 5 (continued)

References	AOPs	Wastewater type	Influent COD (mg L^{-1})	Operation parameters and reduction in pollutants
Kurt et al. (2007)	Electrochemical treatment	Raw tannery wastewater	2810	COD removal (70 %), Electric current (15.0 W), Time (10 min) and pH (3)
Pokrywiecki Sauer et al. (2006)	UV/H_2O_2	Coagulated tannery wastewater	200–800	COD removal (60 %), H_2O_2 (0.5 h L^{-1}), Time (4 h)
Schrank et al. (2005)	Fenton reagent	Coagulated tannery wastewater	130	COD removal (80 %), H_2O_2/Fe^{2+} (500/100 w/w), Time (2 h)
Schrank et al. (2004)	Photocatalysis (UV/TiO_2)	Coagulated/ Flocculated tannery wastewater	2365	COD removal (6 % at pH 3), TOC removal (11 % at pH 3), BOD removal (15 % at pH 7)
Dogruel et al. (2004)	Ozone	Biologically treated tannery wastewater	835	COD removal (30 %), Ozone flow rate (42.8 mg min^{-1}), Time (5 min)
Dantas et al. (2003)	Fenton reagent	Raw tannery wastewater	1803	COD removal (70 %), Time (20 min), pH (2.5) and Temperature (25 °C)

Despite of a broad range of applications, AOPs also have some drawbacks that should also be considered before its applications. The presence of scavenger compounds such as an excess amount of H_2O_2 sometime can act as a hydroxyl scavenger instead of hydroxyl radical source, which interferes with the COD determination and reduces the reaction kinetics making the process uneconomical (Kang 2002; Lofrano et al. 2013). Further, the TWW also contains a significant amount of chromium, which may be oxidized from trivalent to hexavalent form, a more toxic form during oxidation treatment and thus, it is highly recommended to evaluate the possible effects of oxidation on the transformation of chromium atoms in different oxidation states (De Laat et al. 2004; Dogruel et al. 2006; Rameshraja and Suresh 2011; Lofrano et al. 2013). For these reasons, AOPs should be applied more properly to the segregated streams of wastewater containing high amount of aromatic compounds for fenton treatments or high content of salts for electrochemical treatment.

Moreover, AOPs still have not been put commercially at large scale (especially in the developing countries) even upto today mostly because of the relatively high costs. Nevertheless, their high oxidative capability and efficiency make AOPs popular techniques for the tertiary treatment of recalcitrant organic and inorganic pollutants. The increasing interest in wastewater reuse and more stringent regulations regarding the water pollution prevention and control are currently accelerating the implementation of AOPs at large scale.

5.4 Combinatorial Treatment Approaches

In the previous section, various treatment approaches applied for TWW have been discussed. However, these treatment approaches have some serious limitations that need to be addressed further. The presence of residual organics, dyes, and other impurities in TWW even after the biological treatment processes followed by the RO based membrane technologies have been reported as the major drawbacks leading to membrane fouling and finally failure of treatment processes (Srinivasan et al. 2012). Therefore, a combined application of physico-chemical treatment methods with biological treatment methods or various oxidation processes is generally preferred for the effective TWW treatment. Some of the combined treatment methods applied for TWW is presented in Table 6.

Table 6 Combined treatment approaches reported for tannery wastewater

References	Combined treatment applied	Pollutants	Optimum parameters
Suganthi et al. (2013)	Hybrid membrane bioreactor	COD and Color	Electric current density (15 mA/cm^2), Electrocoagulation time (15 min), Membrane area (0.0143 m^2), Membrane spacing (0.22 μm), pH (7.4 and 9)
Srinivasan et al. (2012)	Biological treatment with ozonation	COD and color	Ozone flow rate (3 g/h), Time (24 h), pH (12), Hydraulic retention time (36 h), sludge age (10 days)
Mandal et al. (2010)	Biological treatment with fenton oxidation	COD, BOD, Chromium, Sulphide and Color	Fenton reagent (6 g $FeSO_4$ and 266 g H_2O_2), Time (30 min: fenton oxidation, 72 h: biological oxidation), pH (2.5), Temperature (30 °C)
Iaconi et al. (2009)	SBBR with ozonation	COD, BOD, TSS, TKN and color	Sludge production (0.4 kg TSS/kg COD), Time (5760 and 2160 h)
Rodrigues et al. (2008)	Photo-electrochemical treatment with electrodialysis	COD and NH_4-N	Electric current density (36 mA/cm^2), Ti electrode, Membrane area (1.72 dm^2), Membrane spacing (0.75 mm)
Dogruel et al. (2006)	Biological treatment + ozonation with biological treatment	COD	Ozone flow rate (20 g/h), Reaction time (30 min)
Naumczyk and Rusiniak (2005)	AOP with fenton reagent	COD and Ammonia	Fenton reaction time (30 min)
Szpyrkowicz et al. (2005)	Electrochemical treatment with biological treatment	COD and Ammonia	Sludge production (1.37 kg/m^3/day), Electrolysis time (49 min)

(continued)

Table 6 (continued)

References	Combined treatment applied	Pollutants	Optimum parameters
Kennedy et al. (2004)	CAACO system	COD, BOD, Sulphide and sulfate	Volumetric loading rate (0.7376 m^3/m^3 day), Surface loading rate (0.2438 m^3/m^3/day)
Iaconi et al. (2004)	SBBR with ozone oxidation	COD, TKN and TSS	Sludge production (0.05 kg VSS/kg COD)
Iaconi et al. (2003)	SBBR with ozonation	COD, TKN and TSS	Sludge production (4 kg/kg COD), Organic loading (2.6 kg COD/m^3/day)
Di Iaconi et al. (2002)	SBBR with ozone oxidation	COD, Ammonia and SS	O_3 flow rate (8.7 mg O_3/min), Sludge production (4 kg TSS/kg COD)

6 Waste Minimization, Operation, Treatment and Management in Leather Industries

6.1 Solid Waste Generation, Treatment and Management

In LIs, apart from liquid waste, a large amount of chromium containing tanned solid waste (non-biodegradable sludge) is also generated during leather processing (Dixit et al. 2015). The waste generated finds very limited applications and its disposal causes serious environmental problems (Mwinyihija 2010, 2012). The types and quantity of solid waste generated during the processing of 1 t of raw hide/skins have been presented in Table 7.

However, the conventional treatment and disposal of solid waste is not environmentally feasible because of transformation and leaching of Cr(III) from tanned waste to Cr(VI) and groundwater, emission of nitrogen oxide (NO_x), hydrogen cyanide (HCN) and ammonia (NH_3) (Fathima et al. 2012; Dixit et al. 2015). Therefore, the combination of aerobic treatment (for degradation of low molecular weight compounds) with anaerobic treatment (for further degradation of metabolites) may be a suitable treatment option for tannery waste. The methodologies for the treatment of liquid tannery waste using solid tannery waste have been recently discussed by Fathima et al. (2012). Further, after treatment the remaining waste can be recycled and utilized as useful by products and raw materials. Some of the technological options, which are proposed for the handling and management of solid waste, are presented in Fig. 1.

Table 7 Nature and quantity of solid waste generated during the processing of 1 t of raw hide/skins

Nature of solid waste generated	Quantity (kg)
Salt from handshaking	80
Salt from solar pans (not realized)	220
Hair (pasting ovine)	100
Raw trimmings	40
Lime sludge (mostly bovine)	60
Fleshing	120
Wet blue trimmings (grain splits)	30
Chrome splitting (bovine)	65
Chrome shaving (mostly bovine)	95
Buffing dust (including shaving bovine after crust)	65
Dyed trimmings	35
Dry sludge from CETPs	125

Adapted from Rao et al. (2004) and Thanikaivelan et al. (2005)

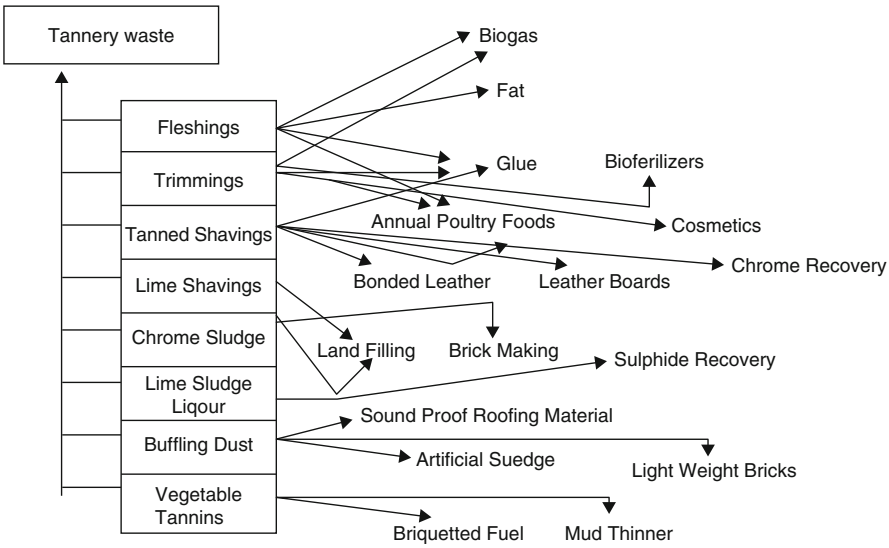

Fig. 1 Technological options for handling and management of solid waste generated during leather production (adapted from ILTIP 2010)

6.2 Gaseous Emission and Control

The emission of gaseous waste such as ammonia (during deliming, unhairing and drying), hydrogen sulphide (released in TWW from sulphides if pH is >8), particulate matter (containing chromium from reduction of chromate or from buffling), and volatile organic compounds (hydrocarbons, amines and aldehydes) from LIs during the different steps of tanning processes may also cause atmospheric pollution (Dixit et al. 2015). Therefore, the proper control of gaseous emission should be required.

6.3 Clean Technologies for Hazards Minimization

Environmental pollution due to LIs is a major cause of concern and its mitigation requires some cleaner technologies (CTs) or also regarded as greener technologies (GTs) for pollution prevention and hazards minimization. CTs utilize the processes that avoid the use of harmful chemicals or promote the use of eco-friendly chemical and cut or eliminate the gaseous emissions and wastes and therefore are cost-effective. Various CTs for the tannery waste minimization and control have been reviewed by many workers (Thanikaivelan et al. 2005; Lofrano et al. 2013; Islam et al. 2014; Dixit et al. 2015).

The development and implementation of CTs at large scale require (a) careful auditing and assessment of the toxicological effects of chemicals used in leather processing, (b) to avoid the use of environmentally susceptible chemicals, (c) to ensure the maximum uptake of chemicals used, (d) assessment of environmental impact of waste generated during leather processing, and (e) optimization of processes for the best economic returns. However, the success of CTs depends on the following parameters: (a) reduction of pollution load in terms of quantity and quality, (b) tanner's benefit in terms of leather quality and/or cost reduction, (c) reproducibility of the process, (d) economic feasibility of process (e) wide market opportunities. Further, the use, assessment and selection of best available techniques (BAT) for the tanning of hides and skins have been discussed (IPPC 2013).

7 International Legislations Scenario for Tannery Wastewater and Chemicals

7.1 Legislations for Discharge Limits of Tannery Wastewater

In developing countries, according to the environmental pollution control regulations set by various national and international environment protection agencies, LIs are forced to set up the WWTPs either individually as ETP or collectively as CETP and the treated wastewater should comply with the discharge standards. The compliance with the discharge standards has not always been practical either because the laws are too ambitious or unrealistic in case of certain parameters, or they have lacked the effective instrumentation and institutional support. Some environment protection laws have not succeeded because they do not match the technical requirements and economic reality of the country or they do not have the institutional support to implement them into consideration.

In India, during the 1990s, several LIs were ordered to close their units as these could not meet the discharge standards, while many of them paid huge compensation for the damage caused due to the groundwater contamination (CSIRO 2001). For the sake of LIs, the Indian government has offered subsidies to construct

Common Effluent Treatment Plants (CETPs) for the treatment of TWW. Notwithstanding, the pollution problems are still common due to high operation and management cost associated with CETPs and thus causing illegal dumping of wastewater (Beg and Ali 2008). In Uganda, the main leather industry was found to dump its wastewater directly into a wetland adjacent to Lake Victoria (The Monitor 2009) whereas in Croatia, the pollution abatement cost exceeded the compensation cost against the irresponsible behaviour of LIs (EcoLinks 2001).

The environmental pollution due to the discharge of TWW has become a serious concern in recent years. For pollution prevention from TWW and its chemicals, the United Nations Industrial Development Organization (UNIDO) has compiled the standard limits for the discharge of TWW into water bodies and sewers from several countries worldwide (UNIDO 2000, 2003). The discharge standards for some of the countries are presented in Table 8. The discharge limits for TWW may vary from country to country and are either related to the quality of treated wastewater or the quality of receiving water bodies (Dixit et al. 2015).

7.2 Legislations for Leather Chemicals

A variety of chemicals are used during the leather processing, which are highly toxic to living beings and cause environmental pollution. In this view, some countries have also made regulations for the production, import and sale of leather products containing harmful chemicals. The chemicals and their permissible limits in leather and leather products approved in some countries are summarized in Table 9. However, the European Chemical Agency (ECHA) has also prioritized and restricted the use of a few chemicals in LIs under Substances of Very High Concern (SVHC), which are considered to be hazardous for environment and human beings (UK REACH 2009). However, all the chemicals are still used in leather making and therefore their proper control is urgently required.

8 Challenges and Future Prospects

Today's the LIs are facing some serious challenges posed by the public and governments mainly due to the environmental pollution and there is a public outcry against the industry. The major challenges faced by LIs include:

(a) Increased cost of leather production per unit area due to the stringent environmental regulations.
(b) Increasing demand of raw material i.e. raw hides, skins and semi-finished leathers.
(c) Lack of advanced processing techniques and waste treatment technologies in developing countries.

Table 8 Discharge limits for tannery wastewater into water bodies and sewers in some countries

S. No.	Parameter	Italy		Turkey		Netherlands		Argentina		Brazil		Egypt		China		Vietnam		Indonesia		Bangladesh		India		Pakistan	
		S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	*S^a	S^b	S^a	S^b	S^a	**S^b
1.	pH	5.5–9.5	5.5–9.5	6–9	6–10	6–10	6.5–10.0	5.5–10	5.5–10	5.0–9.0		6.0–9.0	6.0–9.0	6.0–9.0	6.0–9.0	5.5–9.0	5.5–9.0	6.0–9.0				5.5–9.0	5.5–9.0	6.0–9.0	6.0–9.0
2.	Temperature °C	30–35	30–35		40		40	45	45	<40	40	35	0		35	40	45					40–45	40–45	40	
3.	Conductivity (µS/cm)																								
4.	Suspended solids (mg/L)	40–80	200	150	350	150	350					30	500	70–150	400	100	200	150	150		500	100	600	200	
5.	Settleable solids							0.5	0.5	1.0		[a]	5–10		10										
6.	BOD_5 (O_2 mg/L)	40	250	100	250	5	250	50	200	60		20–30	400	20–100	600	50	100	150	150		250	30	500	80	
7.	COD (mg/L)	160	500	200	800	[a]	[a]	250	700			30–40	700	100–300	1000	100	400	300	300		400	250		150	
8.	TDS (mg/L)											800–1200	2000									2100	2100		
9.	Sulphide (S^{2-}) (mg/L)	1	2	1	2	[a]	[a]		1	0.2	5	1	10	1	10	0.5	1.0				2.0	2	2	1	
10.	Chrome (III) (mg/L)		4				1				5			1.5	2.0	1.0	2.0					2	2		
11.	Chrome (VI) (mg/L)	0.2	0.2	0.3		[a]	[a]							0.5	0.5							0.1	0.1		
12.	Total Chrome (mg/L)	2	4	2	5	0.05	2	0.5	2	0.5		0.05	5–10	1.5	1.5	2.0	2.0	2	2		2.0	2	2	1	
13.	Chloride (mg/L)	1200	1200			200	[a]	[a]	[a]			[a]	[a]									1000	1000	1000	
14.	Sulfates (mg/L)	1000	1000		1700	3		[a]	1000			[a]	[a]									1000	1000	1000	
15.	Ammonia (mg N/L)	10–15	30					3	10	5		100	100					10	10			50	50	40	
16.	TKN (mg N/L)				100	[a]	[a]	10	30	10		[a]	[a]			60	60								

(continued)

Table 8 (continued)

S. No.	Parameter	Italy		Turkey		Netherlands		Argentina		Brazil		Egypt		China		Vietnam		Indonesia		Bangladesh		India		Pakistan	
		S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	S^a	S^b	*S^a	S^b	S^a	S^b	S^a	**S^b
17.	Phosphorous (mg P/L)									1															
18	Oil/grease (mg/L)	20	40	20	100			100	100	20–30	100	100	100	10–15	100	10	30	5	5		20	10	20	10	
19.	Phenol (mg/L)	0.5	1		10	[a]	[a]	0.5	0.5	0.1–0.5		0.001–0.002	[a]	0.5	2.0				1			5–50	5–50	0.3	
20.	Detergents (mg/L)																		1.5						
21.	Solvents (mg/L)																								
21.1.	Hydrocarbons (mg/L)	0.2	0.4																						
21.2.	Nitrogenous (mg/L)	0.1	0.2																						
21.3.	Chlorinated (mg/L)	1	2					1	2	5															

S^a: Surface, S^b: Sewer, *S^a: Bangladesh has no discharge standards for tannery wastewater into surface water, **S^b: Pakistan has no discharge standards for tannery wastewater into sewer

[a]Spaces left blank indicate that parameters which are not specified and considered as specific requirements that need to be fulfilled

Table 9 Maximum permissible limits of chemicals of leather products in some countries (adapted from Dixit et al. 2015)

Residual substances limits for chemicals	European Union	Germany	Austria	Denmark	France	Netherlands	Switzerland
Azodyes[a]	30 ppm						
Pentachlorophenol	30 ppm	5 ppm	30 ppm		30 ppm	30 ppm	30 ppm
Phthalates	0.1 %	0.1 %		0.05 %			
PCBs and PCTs[b]	Not to be used						
Biocides[c]	5 ppm	5 ppm	5 ppm		5 ppm	5 ppm	10 ppm
Hexavalent Chromium	3 ppm	10 ppm					
Cadmium	100 ppm		75 ppm			100 ppm	100 ppm
Arsenic	Nil						
Lead	90 ppm						
Organotin Compounds	Nil						
Specific Flame Retardants	<0.1 %						
Formaldehyde		>1500 ppm	>1500 ppm		200–400 ppm	120 ppm	

[a]Azo dyes: Biphenyl-4-ylamine; 4-aminobiphenyl xenylamine; Benzidine; 4-Chloro-o-toluidine; 2-Naphthylamine; o-aminoazotoluene; 4-amino-2′, 3-dimethylazobenzene; 4-o-tolylazo-o-toluidine; 5-Nitro-o-toluidine; 4-chloroaniline; 4-methoxy-m-phenylenediamine; 4,4′-methylenediani-line; 3,3′-dichlorobenzidine; 3,3′-dimethoxybenzidine o-dianisidine; 3,3′-dimethylbenzidine 4,4-bi-o-toluidine; 4,4-methylenedi-o-toluidine; 6-methoxy-m-toluidine; p-cresidine; 4,4′-methylene-bis-(2-chloroaniline); 4,4′-oxydianiline; 4,4′-thiodianiline; o-toluidine; 2-aminotoluene; 4-methyl-m-phenylenediamine; 2,4,5-trimethylaniline; o-anisidine 2-methoxyaniline; 4-amino-azobenzene

[b]PCBs: Polychlorinated biphenyls; PCTs: Polychlorinated terphenyls

[c]Biocides (23 approved): Human hygiene biocidal products; Private area and public health area disinfectants and other biocidal products; Veterinary hygiene biocidal products; Food and feed area disinfectants; Drinking water disinfectants; Preservatives; In-can preservatives; Film preservatives; Wood preservatives; Fibre, leather, rubber and polymerised materials preservatives; Masonry preservatives; Preservatives for liquid-cooling and processing systems; Slimicides; Metalworking-fluid preservatives; Pestcontrol; Rodenticides; Avicides; Molluscicides; Piscicides; Insecticides, acaricides and products to control other arthropods; Repellents and attractants; Other biocidal products; Preservatives for food or feedstocks; Antifouling products; Embalming and taxidermist fluids; Control of other vertebrates

(d) Lack of specific dedicated industrial areas for the positioning of LIs.
(e) Poor capacity utilization leading to the higher financial cost and overheads charges.
(f) Lack of financial support from government.

The mitigation of these challenges requires the financial support at large scale from the government for the upgradation of LIs, especially small scale industries (Xu and Zhiping 2011). Hence, there is a need to revisit the leather processing again for making the continued sustainability of LIs in near future because LIs are the key drivers of many nation's economy.

9 Summary and Conclusion

(a) LIs are one of the major sources of environmental (soil, water, air) pollution.
(b) TWW is a highly polluted wastewater among all the industrial wastewater.
(c) Currently, the processes used for leather making in several developing countries are traditional and required to be optimized for chemical and water consumption.
(d) The search for some other suitable tanning agents to replace the chromium is urgently required for eco-sustainable tanning process.
(e) Sulfide is highly toxic but the mechanism of toxicity is not well understood and implementation of adequate technology for H_2S desorption is required.
(f) Membrane bioreactors and constructed wetlands are the eco-friendly options for the treatment of TWW and its management, but have some limitations that need to be addressed in the future.
(g) The combinatorial approaches involving physical or chemical with biological treatment process to treat the TWW may give satisfactory results as compared to the individual treatment process.
(h) The emerging treatment approaches like membrane filtration and oxidation processes are also currently using/under analysis.
(i) AOPs are much promising to remove the recalcitrant organic pollutants but there is a still need to optimize these for best economic returns.
(j) The emerging anammox technology for the anaerobic removal of ammonia from TWW is under research and further investigation is required.
(k) A complete understanding of toxicity profiles of TWW may also be helpful in achieving the appropriate treatment solutions for future tanneries.
(l) Locating LIs in a planned industrial area is another common approach to abate the environmental pollution in parallel to strengthen the discharge limits for TWW.
(m) The use of eco-friendly chemicals, water minimization technologies and wastewater treatment/purification and recycling as per the EU integrated pollution prevention strategy and greening policy will be fruitful for solving the environmental problems.

Thus, we can say that there is no treatment method at its best to treat TWW and its chemicals. However, it is clear that continuous efforts are required in order to search for the better treatment approaches for TWW in near future. Further, the emerging treatment approaches like AOPs in combination with biological treatment processes will remain an agenda for the policy makers and water sector professionals to apply the best pollution prevention solution for the future tanneries.

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Disposal of Unused Drugs: Knowledge and Behavior Among People Around the World

Milica Paut Kusturica, Ana Tomas, and Ana Sabo

Contents

1 Introduction

In the last decade, the disposal of unused or expired pharmaceuticals has gained a particular interest. The global increase in pharmaceutical spending has led to an enhanced international awareness on the issue of unused medications and harmful economic, environmental and health effects of improper medicine disposal (Braund et al. 2009; EEA 2010; Kümmerer 2008). Apart from the measures employed to reduce the creation of unused medication and lower the economic burden arising from direct costs of discarded medications (Ruhoy and Daughton 2007),

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countrywide plans for promoting and implementing adequate disposal methods can limit potentially damaging environmental effects (West et al. 2014).

The presence of active pharmaceutical ingredients in the aquatic environment, first attracted attention back in the 1970s, but it wasn't until the introduction of better analytical instruments that the topic began to gain more and more interest among scientific community (Daughton and Ruhoy 2009; Kümmerer 2008). As the analytical techniques continue to improve in precision and accuracy, the number and frequency of detection of trace organic chemicals in the environment, including pharmaceutical products are increasing. All this raised concerns about potential exposure of humans to these chemicals through the drinking water, and to living organisms in surface waters as the pharmaceuticals are difficult to remove with conventional wastewater treatment processes (Tischler et al. 2013; Cook et al. 2012). Several compounds occur frequently in the fresh water environment and may possibly in the mixture pose a human health hazard at environmental concentrations (Murray et al. 2010; Houtman et al. 2013). Pharmaceuticals frequently detected in the freshwater environment include a variety of analgesics, anticonvulsants, anti-epileptics, antibiotics, painkillers and synthetic hormones (Murray et al. 2010). Pharmaceuticals are deposited in the environment following excretion after use or removal of topical medication during bathing and through disposal of unwanted pharmaceuticals. Disposal of discarded medications via environmentally-unfavorable routes such as the sink, toilet or rubbish bin (Braund et al. 2009) are important factors contributing to water and ground contamination and the overall environmental occurrence of pharmaceutically active compounds as these substances are difficult to remove with conventional wastewater treatment processes (Glassmeyer et al. 2009).

To date, quantity and quality of unwanted residential medication, disposal practices as well as the potential causative factors or reasons for medication wastage have been the subject of several studies (Langley et al. 2005; Ekedahl 2006; Guirguis 2010; Braund et al. 2009; Mackridge and Marriott 2007; Vogler et al. 2014; Zargarzadeh et al. 2005; Abou-Auda 2003). There have been two systematic reviews on the issue of medication wastage: one focused on the disposal practices for unused medications (Tong et al. 2011) and the other critically appraised and presented the available data on the possible causative factors associated with medication wastage and the effectiveness of interventions on wastage reduction (West et al. 2014). Considering that there have been 5 years since the last review article on household medication disposal practices, the overall aim of this systematic review was to evaluate critically, summarize and present the available evidence on the current disposal practices for residential unwanted medication and to identify possible connection between environmental awareness and behavior regarding this issue. More specifically, this review pursued to answer the following questions: What are the predominant methods of household drug disposal around the world? Is there an impact of knowledge about environmental effects of improper drug disposal on behavior regarding this practice?

2 Methods

The research for inclusion of articles in this review was performed in October 2015. The search was limited to articles published since January 2005 to the end of September 2015; grey literature and conference abstracts were excluded. The keywords "medication OR medicine OR drugs" AND "disposal OR unused OR wastage" and "household OR residential OR home" were used in searches involving following databases: PubMed, Web of Science, Google Scholar, Scopus and The Cochrane Library. Multi-step approach was used: first selection based on titles, then on abstracts, and then on full-texts. All titles mentioning disposal, unwanted medication or medication wastage were checked. Two persons independently reviewed these articles; all disagreements were reexamined jointly and after appropriate corrections were made reviewers selected the studies to be included in the review. Another researcher reviewed these decisions. Studies were included in this systematic review if they met all of the following criteria: (1) Study explicitly identified medication disposal methods utilized by households or individuals (2) The study clearly articulated their research design, methods and sample size. (3) Study reported quantitative observational or survey data in the form of frequencies, ranks or proportions on methods of drug disposal for unwanted or expired medication from households and/or knowledge or awareness regarding this issue. (4) The study was in English or a translation was available. (5) The study was published in or after January 2005 until October 2015. (6) The study has been published in a peer-reviewed journal. Studies focusing solely on the reasons for the non-use of the medicines, number and types of unwanted or expired medication in households, reasons for returning unused medicines to the pharmacy or studies analyzing contents of a pharmaceutical waste sample without reporting the disposal practices were excluded. Quality assessment was carried out using adapted version of quality assessment scale by Kmet et al. (2004). Reviewers then extracted the following data from studies: country where the study was conducted, year of publication, aim of the study, description of respondents included, study methods, sampling techniques, sample size, numerical data for disposal methods (grouped into four categories: disposal into household garbage, flushed down the sink or toilet, returned to pharmacy or other collection site and other). Reviewers also identified data regarding knowledge about medication wastage, impact of medication disposal, if such information was reported in the study.

3 Results

Figure 1 provides information on the number of articles identified. The search resulted in 830 citations from which relevant studies were selected for the review. Initially, the articles with titles duplicated in the databases were excluded. After removing the duplicates and examination of 584 titles and 223 abstracts, 28 were

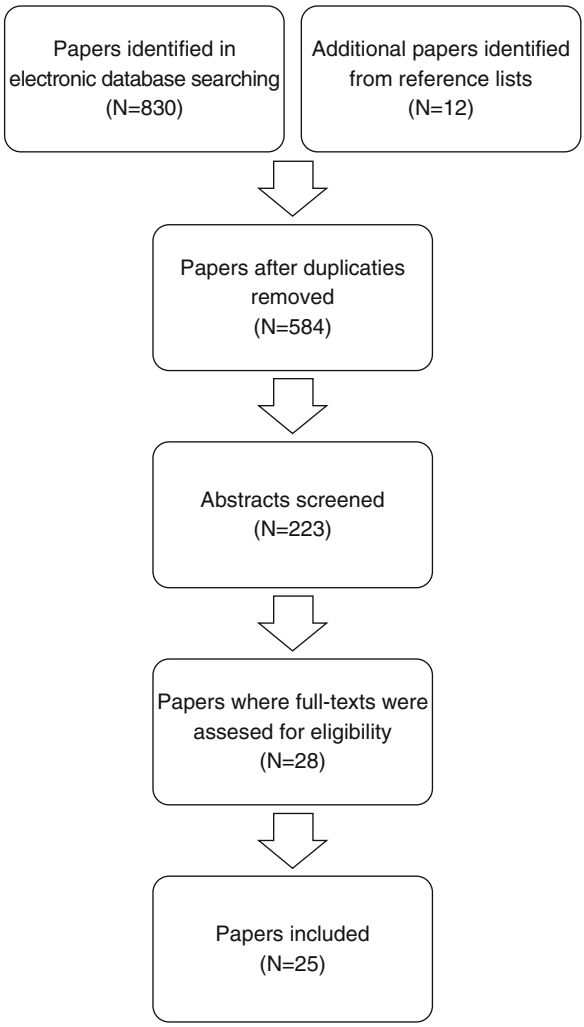

Fig. 1 Diagram of literature retrieval and screening

found to be potentially relevant and their full papers were obtained. Manual search of bibliographies of retrieved references yielded further 12 articles. Out of 40 full texts articles considered, 25 studies met all inclusion criteria. Two studies were not included because they were published in another language and translation was not available (Chen et al. 2012; Pinto et al. 2014). The key data of all surveys is summarized in Table 1. Surveys were conducted in 19 different countries and included a total of 18008 respondents. Seven studies were carried out in Europe (Bound and Voulvoulis 2006; Krupiene and Dvarioniene 2007; Gotz and Keil 2007; Persson et al. 2009; Kusturica et al. 2012; Fenech et al. 2013; Vellinga et al. 2014), five in Middle East (Abahussain et al. 2006; Abahussain and Ball 2007; Kheir et al. 2011; Abdallah et al. 2014; Abdo-Rabbo et al. 2009), three in Africa (Auta et al. 2011, 2012; Sasu et al. 2012), four in Asia (Aditya 2013; Iabu et al. 2013;

Table 1 Studies and their methodologies

Source (year, authors, settings)	Study aim	Research instrument	Sampling method and study population	Sample size
2006 Bound and Voulvoulis UK (Southeastern)	To investigate the household disposal of unused and expired pharmaceuticals as a source of pharmaceuticals in environment	Close ended questions: interview. Asked about disposal methods and types of medications disposed	Interviewers drawn from a representative mix of households in cities, towns spread evenly across age ranges and family types	392
2006 Seehusen and Edwards U.S.	To identify patients' disposal habits and explore patient's beliefs about disposal methods	Anonymous, 22 questions survey on computerized kiosk. The program encouraged all questions to be answered, thereby minimizing missing data	Convenience sample of patients entering the outpatient pharmacy waiting room	301
2006 Abahussain et al. Kuwait	To measure the practice and attitude with regard to safe disposal of unused medicines by Kuwaiti patients receiving prescribed medicines at major public hospitals	Self-administered questionnaire with close-ended questions about disposal methods and opinion of the most appropriate disposal method of unused drugs	Kuwaiti patients visiting the outpatient (ambulatory) pharmacies for prescription dispensing at five large public hospitals	300
2007 Abahussain and Ball Kuwait	To test the effectiveness of a simple educational intervention to encourage households to return unwanted medicines via a municipal collection programs, to determine householders' opinion as to the best method of returning unused medicines and to investigate the most common sources and types of unwanted medicines at home	Self-administered questionnaire that asked participant opinion on household medication disposal and views on views on a proper disposal system	Convenience sample of households equally derived from suburbs of high/middle and middle socioeconomic status	250
2007 Gotz and Keil Germany	To clarify to what extent consumers in Germany dispose of unused medicines directly via domestic sewage	Bias controlled interview delivered on multimedia pen pads	Random selection of households	1306

(continued)

Table 1 (continued)

Source (year, authors, settings)	Study aim	Research instrument	Sampling method and study population	Sample size
2007 Krupiene and Dvarioninene Lithuania	Analyses of pharmaceutical pathways to the environment, paying attention to the disposal patterns and the habits to dispose of unused, unwanted or expired medicines which vary among residents of different living places in Lithuania	Closed-ended postal questionnaire regarding whether they returned unused drugs to pharmacies	Convenience sample of households from towns, suburbs and settlements and the countryside	200
2009 Braund et al. New Zealand	To determine the proportion of unused medications that are not returned to a pharmacy and are disposed of via land fill or water systems and to identify why these medications were unused or unwanted	Closed-questions online survey instituted by the National Poisons Centre	Convenience sampling from anonymous website visitors	452
2009 Kotchen et al. U.S.	To provide information about general awareness of the issue, disposal practices, willingness to pay for a disposal program, and willingness to participate in disposal program	Closed questions regarding medicines disposal, environmental awareness and willingness to pay	Phone survey of randomly selected households	1005
2009 Person et al. Sweden	To learn about what the general public does with unused prescription drugs as well as the pertaining attitudes to this issue. To map the knowledge about correct handling of unused drugs and also to get a view to what extent the information campaigns could raise the awareness and change the behavior among the general public	Closed questions about medicine disposal and weather medicine disposal habits correlated with their attitudes	Phone questionnaire, randomly chosen households were contacted	1000

2009 Abdo-Rabbo et al. Oman	To identify the common problems of medicine use in Oman in order to improve the appropriate use of medicine	Questionnaire composed of closed questions covered many aspects of drug use, including storage, expiry date and disposal of unused medicines	Random sampling of patients or their care-givers on exit from primary health care centers	6675
2011 Kheir et al. Qatar	To explore these relationships by characterizing medications kept in Qatar homes, specifically drug quantity, therapeutic class, sale class, usage, method of storage, method of disposal, and expiry status	Telephone interview with questions about disposal methods	Randomly selected households from telephone directory, nationwide	49
2011 Auta et al. Nigeria	To determine the common classes of unused medications in households and medication disposal practices	Pre-tested questionnaire on unused medication disposal	Stratified sample of households	427
2012 Auta et al. Nigeria	Identifying the types of medicines found in pharmacy students' residence and to determine if a relationship exists between keeping medicines in students' accommodation and self-medication practices	Pretested self-administered questionnaire	Random sampling of pharmacy students	240
2012 Kusturica et al. Serbia	To investigate the storage and disposal habits for medications of the population in the South Backa District of Serbia and to get insight into the attitudes and knowledge of the population with respect to the proper medication disposal practices	Closed-end interviewer-based questionnaire focused on attitude, knowledge and preferred methods of medication disposal	Households randomly selected from the telephone directories	208
2012 Sasu et al. Ghana	Study on pharmaceutical waste management practices at homes and hospitals in Ghana	Interviewer-based questionnaires and personal observation	The general public, spread across all age groups and employment classes	83

(continued)

Table 1 (continued)

Source (year, authors, settings)	Study aim	Research instrument	Sampling method and study population	Sample size
2013 Iabu et al. Bangladesh	To evaluate the understanding, public awareness and manners to develop about harmful effects on medication's waste	The students answered the questions about awareness, method of disposal, reasons, knowledge and sources of the students on practice of unused medications	The questionnaires were distributed into four different years of study to students of pharmacy department	290
2013 Fenech et al. Malta and Ireland	An understanding the current of current disposal practices and the relevance of the various routes of unused entry into the environment	An online survey of current attitudes and practices related to the use and disposal of medication	–	1130
2013 Aditya India	To explore the knowledge, attitude, beliefs about drug wastage and methods adopted by students to dispose of unused and leftover medications at home	Self-administered structured questionnaires	Undergraduate dental students	244
2013 Wieczorkiewicz et al. U.S.	To determine how residents in Cook Country, Illinois, use, store and dispose of their medications to assess the possible impact of these medications on health care and environment	Telephone interviews of the use, storage and disposal of medication	Random sample of households using numbers generated from random digit dialing	445
2014 Vellinga et al. Ireland (Galway and Cork)	To establish baseline information on storage and disposal of medicines	Self-administered or interviewer administered questionnaires	People in the streets were approached randomly, respondents were Irish residents, over 18 years of age with fluency in English	398
2014 Fatokun Malaysia	To examine the pattern of antibiotic use and practices among individuals in a Malaysian community and to identify the variables associated with the likelihood of non-compliance with a course of antibiotic	Cross-sectional interviewer-administered questionnaire	Convenience sample of residents recruited near housing areas, supermarkets, minimarkets, school areas, and shop houses	250

2014 Law et al. U.S.	To examine the extent, amount, type, cost and reasons for unused medications among US households	In the first phase, web based survey with open-ended questions, in the second phase paper based survey	Cross-sectional two phased study using a convenience sample. In the first phase survey using open-ended questions was conducted to the campus community, in the second phase survey was conducted at drug take back events	238 and 68
2014 Abdallah et al. Saudi Arabia	The study focused on the household medicine disposal practices currently used in the Western Region of Saudi Arabia among university students	Close ended questionnaire, self-administered	A cross-sectional research design using non probability convenience sampling. Respondents were university students currently studying in any of the three universities in the western region of KSA	1446
2014 Arkaravichien et al. Thailand	To determine what Thai people do with their unwanted medicines and considered in aspects of impact that might have on the environment	Structured interviews	Study samples were grouped into four clusters according to the villages and random sampling was conducted	311
2014 Reddy et al. U.S.	To determine the patterns of storage, utilization, and disposal of opioids among cancer outpatients	Self-administered questionnaire	Cancer patients who previously received a prescription of opioids and subsequent opioid education from the palliative care team were included in the study	300

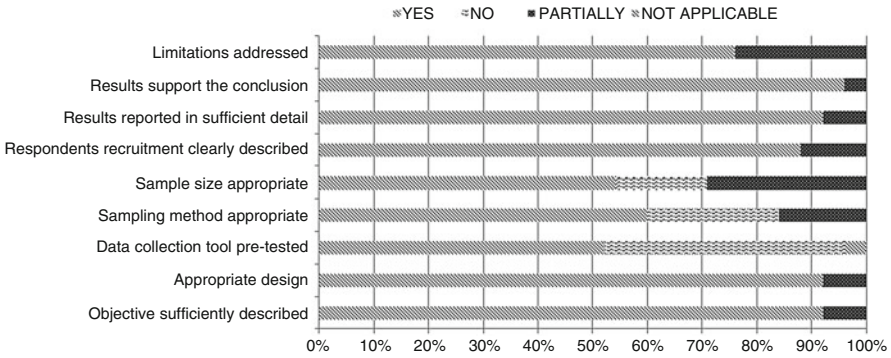

Fig. 2 Quality assessment of 24 studies (100 %)

Fatokun 2014; Arkaravichien et al. 2014), one in New Zealand (Braund et al. 2009) and five in North America (Seehusen and Edwards 2006; Kotchen et al. 2009; Wieczorkiewicz et al. 2013; Law et al. 2014; Reddy et al. 2014). One study had a multi-national sample (Fenech et al. 2013).

Quality assessment was performed for 24 studies; no studies have been excluded on the basis of quality. Details of the assessment of these 24 studies (100 %) are given in Fig. 2. Majority of studies stated a clear research aim, except the ones carried out in Oman and Bangladesh (Iabu et al. 2013; Abdo-Rabbo et al. 2009). The study in Bangladesh did not precisely state the research aim, just that it is essential to investigate all aspects of pharmaceutical use and disposal (Iabu et al. 2013). The study in Oman aimed to assess the knowledge, attitude and practice of patients about medication use in order to identify the common problems in the community, but did not precise what aspects of medication use will be studied (Abdo-Rabbo et al. 2009). Eight studies did not use a pre-tested data collection tools (Bound et al. 2006; Seehusen and Edwards 2006; Abahussain and Ball 2007; Braund et al. 2009; Kotchen et al. 2009; Sasu et al. 2012; Iabu et al. 2013; Aditya 2013; Vellinga et al. 2014). All studies were of cross-sectional design (collected data from a representative sample at one specific time point) and the range of research instruments was used to collect the data: postal survey (1), self-administered questionnaires (10), interviews (4), telephone surveys (4), online and computerized survey (5). One study employed both interview and self-administered questionnaire (Vellinga et al. 2014). The sample size and study populations varied between the countries. The smallest sample was recorded in Qatar and involved only 49 households (Kheir et al. 2011). The largest sample was reported in Oman, and involved 6675 respondents (Abdo-Rabbo et al. 2009). Twelve studies employed probability sampling–nine used random sampling, mostly via telephone directory; (Gotz and Keil 2007; Kotchen et al. 2009; Persson et al. 2009; Kheir et al. 2011; Abdo-Rabbo et al. 2009; Auta et al. 2011; Kusturica et al. 2012; Wieczorkiewicz et al. 2013; Vellinga et al. 2014), two used stratified sampling (Bound et al. 2006; Auta et al. 2012) and one study used cluster sampling

(Arkaravichien et al. 2014). Eight studies used convenience sampling (Seehusen and Edwards 2006; Abahussain and Ball 2007; Krupiene and Dvarioniene 2007; Braund et al. 2009; Fatokun 2014; Law et al. 2014; Abdallah et al. 2014; Reddy et al. 2014). Five studies did not precisely state the sampling method (Abahussain et al. 2006; Fenech et al. 2013; Iabu et al. 2013; Aditya 2013; Sasu et al. 2012). Eleven studies were performed on a sample of households (Bound et al. 2006; Abahussain and Ball 2007; Gotz and Keil 2007; Krupiene and Dvarioniene 2007; Kotchen et al. 2009; Persson et al. 2009; Kheir et al. 2011; Auta et al. 2011; Kusturica et al. 2012; Wieczorkiewicz et al. 2013; Arkaravichien et al. 2014). Four studies were performed on a sample of patients visiting hospitals or pharmacies (Seehusen and Edwards 2006; Abahussain et al. 2006; Abdo-Rabbo et al. 2009; Reddy et al. 2014). The participants involved in studies conducted in Bangladesh (Iabu et al. 2013), India (Aditya 2013), Nigeria (Auta et al. 2012) and Saudi Arabia (Abdallah et al. 2014) were university students. Two studies involved a sample of residents obtained by randomly approaching people on the street (Vellinga et al. 2014; Fatokun 2014). The study from Ghana did not specify the study population, other than it included general public (Sasu et al. 2012). Study by Law et al. involved surveying the households in the first phase, and respondents recruited at the medication take-back event in the second phase (Law et al. 2014).

The methods reported for disposal of unused drugs and attitude or knowledge about medication disposal are summarized in Table 2 and showed on Fig. 3. Based on the results of the reviewed studies, most common method for disposal of unused medications in households is disposal in the garbage. The prevalence rates for disposing of unwanted medication into household garbage ranged from 3 % (Persson et al. 2009) to 100 % in Nigeria (Auta et al. 2011). This is also the leading method of disposal reported in the studies performed in the United Kingdom, Kuwait, Lithuania, Qatar, Serbia, Ghana, Bangladesh, Malta, Ireland, India, Malaysia, Saudi Arabia and Thailand. According to the results reported in studies from New Zealand, USA, Malta, Ireland and Bangladesh disposal of drugs into the sewage system is still common practice, mostly reserved for liquid dosage forms. In Malta and Ireland, respondents stated flushing down the drain 28 % of liquid compared to only 14 % for solid formulations (Fenech et al. 2013). Similar situation was observed in Bangladesh (58 % liquid, 5.1–16 % solids or semisolids) and New Zealand (55 % liquid, 19 % solids) (Braund et al. 2009; Iabu et al. 2013). In the survey conducted in USA in 2006, more than one half of participants had reported flushing medications down the toilet (53.8 %). The survey conducted in Lithuania reported that 50 % of countryside residents dispose of unused drugs by burning them with the rest of the household waste. The practice of burning unused drugs was also reported in Ghana (>10 %) and Nigeria (9.9 %) and in rural households in Serbia (14.9 %). In Sweden, majority of participants reported storing their unused medications or returning them to a pharmacy while flushing unused medications down the toilet or sink has not been reported. Lack of adequate information on desirable methods of residential medication disposal was recorded in many surveyed countries (Scotland, USA, New Zealand, Bangladesh, Malta and

Table 2 Unused medication disposal practice and attitude or knowledge about medication disposal around the world

Authors, year, country	Settings, formulations or type of drugs (if any)	Disposal methods				Attitude, knowledge
		Return to pharmacy	Rubbish bin	Flush down the toilet or sink	Other (stated)	
2006 Bound and Voulvoulis UK	Total		63.2 %	11.5 %	3.5 % (waste sites or other disposal facility)	For the people who agreed or strongly agreed that they lead an environmentally friendly life, the proportion of pharmaceuticals thrown in the bin (40.8 %) was considerably smaller than those who had no view (71 %) and those who disagreed (66.2 %). While the majority of respondents (94.9 %) agreed that pharmaceuticals could be harmful to your health if misused, just over half felt that they could be harmful to the environment. Around half of all respondents agreed that the environmental toxicity of pharmaceuticals could cause a threat to plants and fish. Antibiotics (76.6 %), hormones (73.6 %) and antidepressants (69.4 %) were perceived to be the most hazardous to the environment
	Antibiotics	18.5 %	71.4 %	10.9 %	1 %	
	Beta-blockers	16.7 %	66.7 %	16.7 %		
	Antidepressants	33.3 %	66.7 %			
2006 Seehusen and Edwards U.S.	Patients have disposed in the past with following methods	22.9 %	– –	53.8 % toilet 35.2 % sink	14 % (returned to a healthcare provider) 11 % (give to friends or family)	Less than 20 % reported ever having been given advice about proper medication disposal. Appropriate method of disposal stated by the respondents: 66.7 % return to pharmacy, 35.7 % flushing down the toilet, rinsing down the sink 21 %

2006 Abahussain et al. Kuwait		11.9 %	76.5 %	11.2 %	8.5 % (give to a friend)	54 % thought that returning drugs to a local pharmacy would be appropriate, 21 % thought there should be special collection containers at local shopping cooperatives while 15 % stated that medicines should be collected from homes by the municipality
2007 Abahussain and Ball Kuwait		–	97 %	2 %	0.5 % (give to a friend)	45 % of respondents think that most appropriate method to dispose of unused medication is in special plastic bags collected from the home by municipality
2007 Gotz and Keil Germany		29 % (always), 11 % (usually), 15 % (sometimes), 11 % (rarely) 33.7 % (never)	6.5 % (always), 9.4 % (usually), 14.3 % (sometimes), 13.1 % (rarely) 56.7 % (never)	Solid medications 1 % (always) 2 % (usually) 7 % (sometimes) 6 % (rarely) 84.3 % (never) Liquid medication 10 % (always) 8 % (usually) 13 % (sometimes) 12 % (rarely) 56.6 % (never)	23 % (recycled alongside cardboard and plastics), 15 % (toxic waste bins)	–
2007 Krupiene and Dvarioninene Lithuania	Towns	3 %	89 %	8 %	2 % (burning)	73 % of the study sample did not think pharmacies were able to take back unwanted medications
	Suburbs	–	87 %	6 %	12.5 % (burning)	
	Countryside	–	50 %	–	50 % (burning)	

(continued)

Table 2 (continued)

Authors, year, country	Settings, formulations or type of drugs (if any)	Disposal methods				Attitude, knowledge
		Return to pharmacy	Rubbish bin	Flush down the toilet or sink	Other (stated)	
2009 Braund et al. New Zealand	Liquid medicines	17 %	24 %	55 %	0.7 % (give away or burn)	Almost a quarter of respondents indicated that had kept unused medications because they were not sure how to dispose of them
	Tablets and capsules	24 %	51 %	19 %	2.4 % (give away or burn)	
	Ointments and creams	13 %	80 %	<1 %	2.4 % (give away or burn)	
2009 Person et al. Sweden		43 %	3 %	–	55 % (kept medicines)	85 % of respondents believed that the right handling of unused drugs was to return it to a pharmacy 42 % were worried of drugs' effects on the environment
2009 Abdo-Rabbo A et al. Oman		12 %	45 %		41 % (keep for future use)	–
2009 Kotchen et al. U.S.		6 %	45 %	28 %	5 % (hazardous waste center)	Respondents that are aware of the issue are less likely to dispose of unused medications in the trash or sink/toilet
2011 Kheir et al. Qatar		–	65 + 12 %	6 %	4 + 12 % (kept medicines)	–
2011 Auta et al. Nigeria		–	100 %	–	–	–

2012 Auta et al. Nigeria		–	72.7 %	4.5 % sink 12.9 % toilet	9.9 % (burn)	–
2012 Kusturica et al. Serbia	Urban Rural	4.8 % 4.3 %	85.6 % 74.5 %	8.7 % 6.4	1 % (burn or give to a friend) 14.9 % (burn or give to a friend)	One-half of the population considered throwing medications in the garbage or toilet to have a detrimental effect on the environment
2012 Sasu et al. Ghana		<5 %	>80 %	<5 %	>10 % (burning)	Only a quarter of the respondents think pharmaceuticals have some impact on the environment. The majority of the respondents either did not know (44.6 %) or responded no (30.1 %)
2012 Azad et al. Malaysia	Liquid medicines Solid medicines Semisolid medicines					
2013 Iabu et al. Bangladesh	Liquid medicines	1 %	17.2 %	58.2 %	22.7 % (give away or no answer)	Most of the respondents (67.3 %) didn't know about medication waste or about drug take back system (96.2 %)
	Solid medicines	3 %	72.8 %	5.1 %	17.2 % (give away or no answer)	
	Semisolid medicines	11 %	33.1 %	16.2 %	37.9 % (give away or no answer)	
2013 Fenech et al. Malta and Ireland	Liquid Pills	<10 %	57 % 68 %	28 % 14 %	–	7 % of Maltese respondents and 21 % of Irish respondents have ever been advised on the best way for medication disposal

(continued)

Table 2 (continued)

Authors, year, country	Settings, formulations or type of drugs (if any)	Disposal methods				Attitude, knowledge
		Return to pharmacy	Rubbish bin	Flush down the toilet or sink	Other (stated)	
2013 Wieczorkiewicz et al. U.S.		11 %	59 %	31 %	Return to physician's office 5 %, return to hazardous waste collection facility 8 % give to someone 6 % never dispose 17 % other 5 % (unspecified)	81 % never received information about proper methods of medication disposal. 75 % respondents stated that flushing down the sink or the toilet was an inappropriate disposal method. Most believed that disposing medications in a secured lockbox located in a pharmacy or physician's office was the best method (65 %); respondents had the least confidence (13 %) in a mail-back program
2013. Aditya India	Solid Semisolid Liquid		92 % 94 % 74 %	2 % / 18 % sink 3 % toilet	6 % 6 % 5 %	Attitudes toward adequate disposal methods: 48 % return to, 41 % garbage, 8 % toilet or sink, 3 % give to family or friends
2014 Vellinga et al. Ireland (Galway and Cork)			51 %	29 % sink 14 % toilet	6 %	19 % of the respondents indicated that they received this advice on what to do with left/over drugs
2014 Fatokun Malaysia	Antibiotics	6.4 %	78.8 %	2.0 %	12.8 % (fridge, give to a friend, leftovers)	–
2014 Law et al. U.S.		11.2 %	62.7 %	18 % toilet	17.4 % store medication	71 % preferred pharmacy as a location for medication disposal compared to other choices, such as fire station, police department, hazardous waste facility
		1.8 % return to physician's office		4.3 % sink	8 % unspecified	

2014 Abdallah et al. Saudi Arabia	Liquid medicines	2.4 % (pharmacy) 2.8 % (doctor)	71.7 %	20 %	2.8 %	60 % of respondents believed that discarded medicines may have a harming impact on the environment. Only a quarter of the respondents said that they have previously asked or received information from a person works in a medical field
	Solid medicines	1.7 % (pharmacy)	63.4 %	31.9 %	1.9 %	
		1.1 % (doctor)				
2014 Arkaravichien et al. Thailand	Liquid	0 %	64.6 %	7.4 %	28.0 % (never discarded) 16.0 % (never-discarded)	–
	Solid	1 %	81.4 %	0 %	1.6 % (land filled) 32.8 % (never discarded)	
	Semisolid	0 %	66.6 %	0 %	0.6 % (land filled)	
2014 Reddy et al. U.S.	Opioids	8 %	17 %	18 %	97 % (do not dispose)	26 % of the respondents are aware of any proper disposal methods for opioids

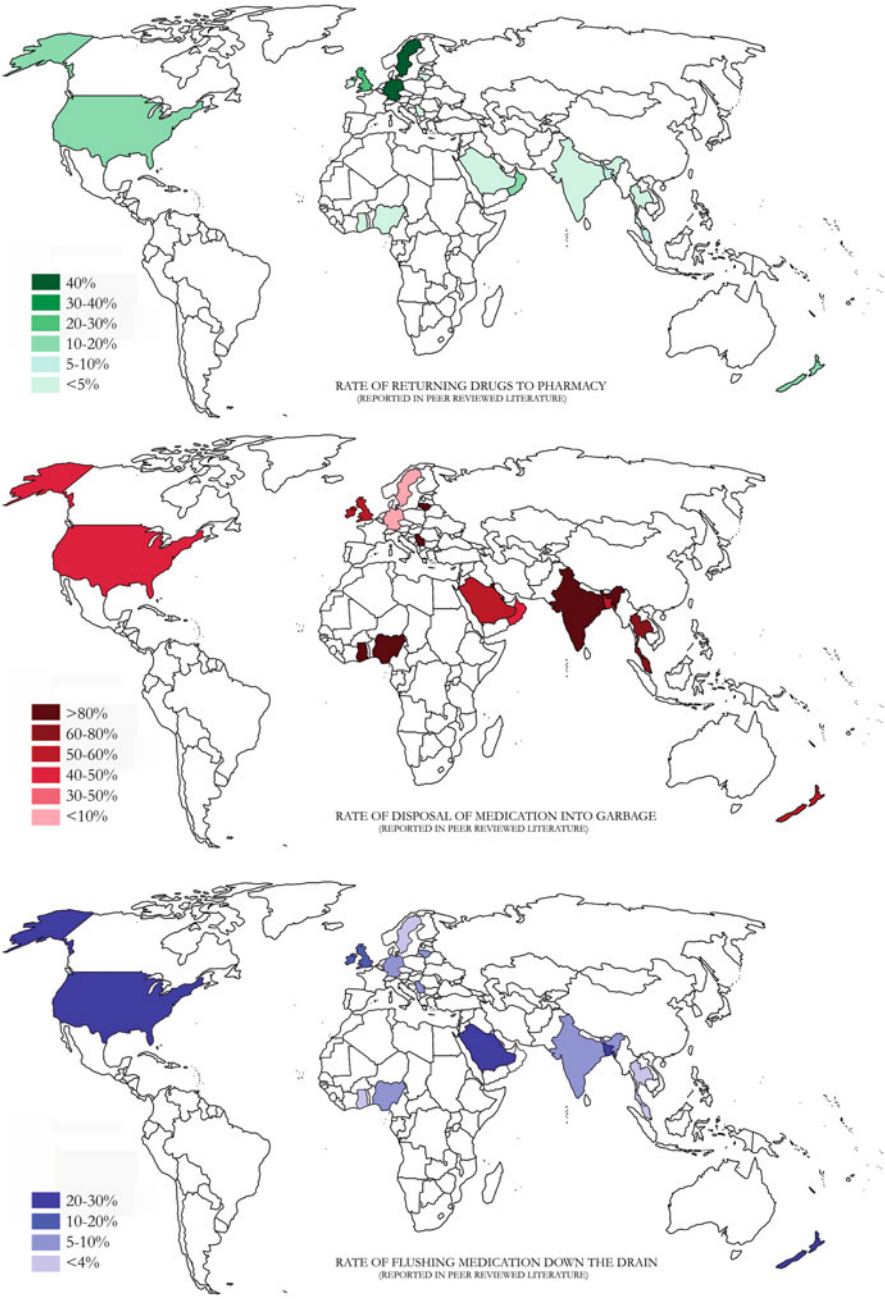

Fig. 3 Methods of drug disposal and frequencies in which they are reported in different countries

Ireland). Although concern on detrimental environmental effects of improper medication disposal was widely present among the respondents, the awareness often did not equate the most common methods of unused medication disposal (Serbia, USA, Kuwait, Malta and UK) (Figs. 4 and 5).

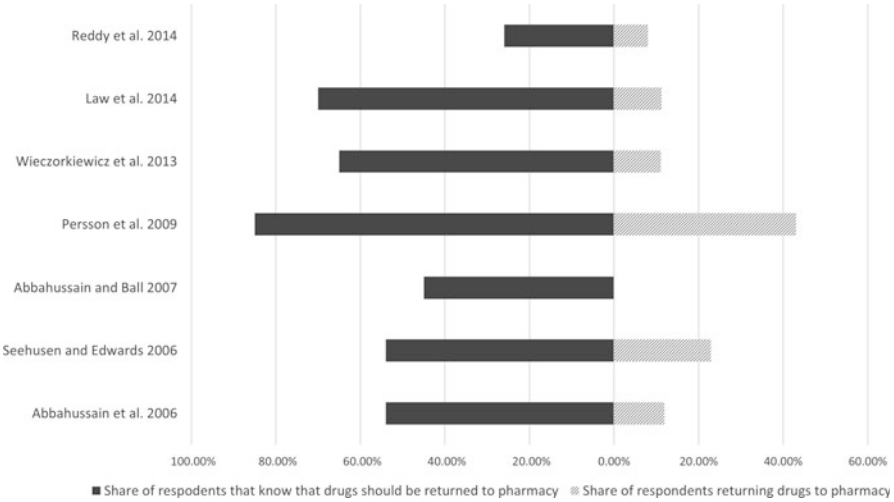

Fig. 4 Rates of reporting knowledge about adequate disposal methods and practice of returning drugs to pharmacy

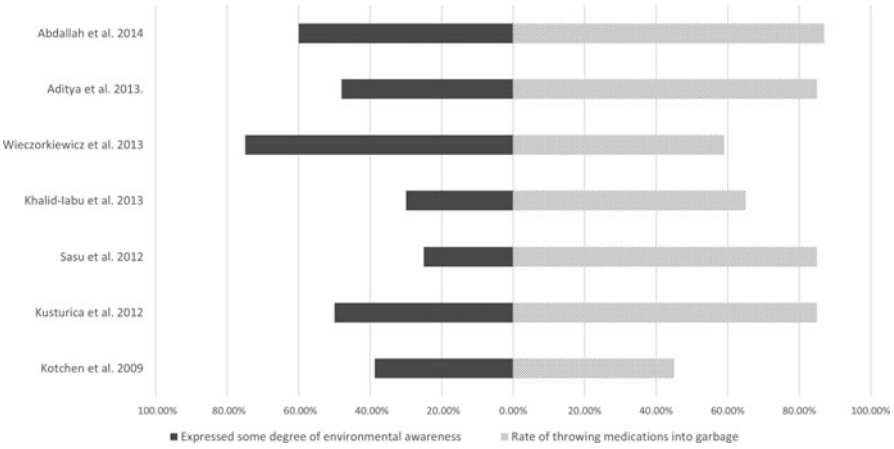

Fig. 5 Rates of respondents reporting environmental awareness and practice of throwing medication into garbage

4 Discussion

This systematic review, which focused on the disposal practices of unwanted medication from general public reported in the peer reviewed literature, included a limited number of studies with varied research designs and populations. Majority of studies were performed by surveying households or general public (Bound et al. 2006; Abahussain and Ball 2007; Gotz and Keil 2007; Krupiene and Dvarioniene 2007; Kotchen et al. 2009; Persson et al. 2009; Kheir et al. 2011; Auta et al. 2011; Kusturica et al. 2012; Wieczorkiewicz et al. 2013; Arkaravichien et al. 2014; Vellinga et al. 2014; Fatokun 2014), but several studies were performed on a sample of dentistry or pharmacy students (Iabu et al. 2013; Aditya 2013; Auta et al. 2012; Abdallah et al. 2014) which makes direct comparison difficult. The studies that were carried out at the drug take-back events or the studies carried out in pharmacies and hospitals (Law et al. 2014; Seehusen and Edwards 2006; Abahussain et al. 2006; Abdo-Rabbo et al. 2009; Reddy et al. 2014) may carry a risk of bias, since completing the questionnaire in the presence of a pharmacist or doctor may have led respondents to provide answers which they thought were 'correct' rather than their actual perceptions or practice. Studies that randomly approached patients in the waiting rooms, or people on the street, could not provide accurate response rate (Seehusen and Edwards 2006; Vellinga et al. 2014; Fatokun 2014; Abahussain et al. 2006) and can also result in a bias as this method of enrolling participants favors certain population groups, due to researcher's selection of particular population. Similarly, several studies (Seehusen and Edwards 2006; Abahussain and Ball 2007; Krupiene and Dvarioniene 2007; Braund et al. 2009; Fatokun 2014; Law et al. 2014; Abdallah et al. 2014; Reddy et al. 2014) employed convenience sampling, and may have introduced a selection bias. The studies using web-based surveys (Braund et al. 2009; Law et al. 2014; Fenech et al. 2013) may have focused more on public which uses computers and internet most often—the relatively young, highly educated people. Study in Qatar had a low response rate using telephone and small sample size so the results of this exploratory study should be considered with caution (Kheir et al. 2011). Also, the study conducted in Ghana did not state how the participants were selected, except that the respondents were spread across all age groups and employment classes (Sasu et al. 2012). Majority of the studies present the self-reported data, which is known to be subjected to recall and non-response bias. Nonetheless, all of these studies provided insight into area of scant knowledge: management of pharmaceutical waste in households, especially in developing countries.

4.1 Methods of Unwanted Residential Medication Disposal

On the basis of the data outlined in this review, the practice of disposing of leftover medicines into the garbage is far more widespread than anticipated and represents

a worldwide phenomenon as the studies carried out in Europe (Bound et al. 2006; Fenech et al. 2013; Krupiene and Dvarioniene 2007; Kusturica et al. 2012), Middle East (Abahussain and Ball 2007; Kheir et al. 2011; Abdallah et al. 2014), Asia (Aditya 2013; Iabu et al. 2013; Fatokun 2014; Arkaravichien et al. 2014), New Zealand (Braund et al. 2009) and in African countries of Ghana and Nigeria (Sasu et al. 2012; Auta et al. 2011, 2012) all reported rubbish bin as the predominant method of disposal for unwanted residential medication. If the trash is incinerated, this may be the environmentally safe way of medication disposal, but if the waste is landfilled, this only delays the entry of pharmaceuticals to aquatic environment. In the case of the household waste being taken to the landfill sites, pharmaceutically active compounds enter the landfill effluent, possibly contaminating surrounding surface or ground waters (Kümmerer 2008; Masoner et al. 2014). The biggest environmental impact of medication disposal into garbage can be expected in countries where landfilling is a predominant (if not the only available) option for municipal waste management. In the EU, landfilling has been defined as the least desired waste management option and many Member States have taken measures to eliminate it completely (landfilling rates for municipal waste had fallen below 1 % in Germany and Sweden). Germany and Sweden rely mostly on incineration (35 % and 56 %, respectively) and recycling (47 % and 33 %, respectively). In some Member States large share of waste is landfilled (65 % in Lithuania, 88 % in Malta, 42 % in Ireland and 35 % in UK) (Eurostat 2014), however, all landfills within EU must comply with EC stringent technical requirements for waste and landfills of Directive 1999/31/EC. This directive aims to prevent or reduce as far as possible negative effects on the environment, in particular on surface water, groundwater, soil, air, and on human health from the waste landfilling. In Serbia, all municipal waste is landfilled, and landfills still do not comply with EU regulations. In the US all landfills constructed since 1994 must meet stringent leachate-control requirements. All municipal solid waste landfills must comply with the federal regulations in 40 CFR Part 258 (Subtitle D of RCRA) (USEPA 2011). Recent US study concluded that the disposal of unused medications in municipal solid waste landfills which meet strict US regulations, effectively eliminates the unused medicine contribution of pharmaceuticals to surface waters as greater than 99.9 % of pharmaceuticals disposed of in a landfill are permanently retained (Tischler et al. 2013).

On the contrary, in some developing countries, large share of all the urban solid waste remains uncollected and the waste that is collected is taken to the poorly operated landfills, which are a significant source of pharmaceutical discharge into environment. In Ghana and Nigeria, only around half of waste generated is collected and disposed of at the landfill and many people simply dump their solid wastes (Owusu et al. 2011; Ugwuh 2009; USEPA 2010). Similarly, Bangladesh and India have minimal waste collection coverage which forces majority of the waste to be dumped in open lands (Zerin and Ahmed 2009; Enayetullah 2006; Bhuiya 2007). In Malaysia and Thailand, landfilling is the only method used for the disposal municipal solid waste, and most of the landfill sites are open dumping areas (Sreenivasan et al. 2012; Manaf et al. 2009; Chiemchaisri et al. 2007). In Middle

East, solid waste management scenario in marked by lack of collection and disposal facilities as authorities are faced with serious challenges emanating from the vast expansion in urban developments, the increasingly unsustainable life style and consumption patterns. In Quatar, more than 80 % of all waste generated nationally ended up in landfills (Al-Maaded et al. 2012). In Kuwait and Saudi Arabia, landfills act as dumpsites, rather than engineered landfills (Omar et al. 2013). Also, disposal of wastes through landfilling is becoming more difficult because existing landfill sites are filling up at a very fast rate. At the same time, constructing new landfill sites is becoming more difficult because of land scarcity and the increase of land prices and high demands, especially in urban areas due to the increase in population (Iabu et al. 2013).

The least favorable route of drug disposal is disposable via drain, leading to direct input of pharmaceuticals into the aquatic environment, with unpredictable acute and especially chronic effects of active compounds, their mixtures and metabolites on environmental organisms. Flushing medication down the drain was mostly reserved for liquid dosage forms, demonstrating the impact of type of formulation on disposal practices. Respondents were 2–5 times more inclined to flush or spill down the drain liquid formulations compared to tablets, capsules and other solids or semisolids. This is universal problem worldwide as different studies, while using different research methods in various demographic and geographical settings all yielded consistent results. This has been reported in studies from Malta and Ireland, Bangladesh, New Zealand and USA (Fenech et al. 2013; Braund et al. 2009; Iabu et al. 2013; Seehusen and Edwards 2006). However, we must mention that the USA survey addressed only methods which directly release pharmaceuticals into the aquatic environment; thus it was unknown how many participants would have disposed of their medications into the rubbish (Seehusen and Edwards 2006). Recent studies carried out in U.S. reported 20–30 % of participants disposing medication via drain (Kotchen et al. 2009; Wieczorkiewicz et al. 2013; Law et al. 2014). It is important to note that current FDA guidelines endorse flushing down the toilet as the suitable disposal method for 23 extremely dangerous controlled substances as flushing down the drain limits potential exposure of children or pets and risks of accidental poisoning, which might have contributed to considerable quantities of flushed medication in American households (FDA 2015).

Home backyard burning of expired and unused medication was commonly reported mean of disposal in Lithuania for respondents living in the countryside (Krupiene and Dvarioniene 2007). In Lithuanian countryside households, 50 % of the respondents reported burning medication compared to only 12.5 % of those living in suburban or urban areas. Similarly, Kusturica et al. reported higher rate of medication burning in rural (14.9 %) than in urban households (1 %) in Serbia (2012). The practice of burning unused dugs was also recorded in Ghana and Nigeria. In fact, the burned pharmaceuticals do not reach the environment as pharmaceutical substances; nevertheless burning of waste in ordinary households burning facilities produces various other contaminants that may be released into the air causing a risk to human health and environment (Kruopienė and

Dvarionienė 2010). It is also interesting to note that no one surveyed in the Lithuanian countryside utilized the sewage system as a mean of disposal of unwanted medications (because of the unavailability of working sewage systems in many parts of the country).

4.2 An Overview on Available Legal Framework and Take Back Programs

An effective collection scheme for medicines whose main purpose is to provide an easy method for disposal of unwanted human medicines could represent an important measure to protect the environment. Under the provisions of current European Union legislation (EC 2004), all EU Member States must establish collection schemes to recover and safely dispose of unused and expired medicines (Directive 2001/83/EC, Directive 2004/27/EC). Article 127b of the European Union Directive requires that "Member States shall ensure that appropriate collection systems are in place for medicinal products that are unused or expired". The approaches used for collection vary among countries, but in general, pharmacies play a central role. The financial support for drug collection also differs among the countries. While some countries rely only on government funding, other are supported through the pharmaceutical industry or the pharmacies themselves (Glassmeyer et al. 2009). However, several surveys have noted that the implementation of these systems and their efficiency varies widely across Member States (Volmer 2010). For example, pharmacies in the United Kingdom are obliged to take back and sort unwanted or unused medicines brought by patients and return these to the National Health Service (NHS) (The National Health Service 2013). On certain occasions, besides the collections in pharmacies, local collection events are also organized by the NHS. Local authorities, without the participation of the pharmaceutical industry, finance the disposal system and promote awareness campaigns. On the contrary, the system for collection of unused medicines in Lithuania is still not fully functioning. According to Lithuanian national legislation, pharmaceutical waste must be collected separately and treated in accordance with the waste management regulation. However, the current regulation does not cover household pharmaceutical waste and it is not clear who is legally responsible. At the same time, all community pharmacies are obliged by law to accept unused and expired medicines and transfer them to a licensed pharmaceutical waste management company every 3 months (Farmacijos istatymas nustato 2006). The legislation states that the government is responsible for the financing of the system but the role of the different institutions is not clearly defined. This means that pharmacies are currently responsible for paying for the disposal of collected medicines and also for communication campaigns. In practice, this means that pharmacies do not communicate widely about their obligation to take back unused pharmaceuticals and sometimes refuse to accept them because of the costs of communication and

disposal (Andriejauskaite and Mikalciute 2008). Among European countries, Sweden is an excellent example of how communicating a message to the public and having unambiguous obligation of specified agencies to accept unwanted medicines for disposal plays crucial part of proper implementation of clearly defined mechanism for disposal. However, the Sweden unused drugs take-back program, where returned medication is collected at the pharmacies, transported to incineration centers and burned under supervision, dates back to 1971, and this certainly contributes to a high percentage of Swedish people returning unused drugs to a pharmacy (Persson et al. 2009).

In the United States, several federal agencies have jurisdiction over different aspects of pharmaceutical regulation. Food and Drug Administration (FDA) protects public health by assuring the safety, efficacy and security of drugs, while Drug Enforcement Administration (DEA) enforces the controlled substances laws and regulations to ensure medications are not diverted for improper uses. The U.S. Environmental Protection Agency (USEPA) protects the environment and human health from chemical exposure via a series of acts. FDA offers consumer information about safe practices for medication disposal on its Website (http://1.usa.gov/1t9icrt). The safest way for drug disposal is a local or national drug take-back program on a national Drug Enforcement Administration (DEA)-authorized collection site. The DEA has periodically sponsored a National Prescription Take-Back day to collect unused medications (Glassmeyer et al. 2009). Additionally, the DEA has authorized certain entities (such as retail pharmacies, drug manufacturers, drug distributors) to become collection sites for leftover medications. If the consumers are not able to travel to DEA collection sites, they may also mix medications with unpalatable substances, place in closed containers, and dispose of the mixture in their household trash. For drugs that carry a high risk of causing harm from accidental ingestion, the FDA recommends they should be flushed down the toilet. In contrast, The Environmental Protection Agency (EPA) does not agree with FDA assertions about the safety of flushing medications, since drug molecules can pass through treatment systems and enter river or lakes, groundwater and even community drinking water (Aschenbrenner 2015).

In New Zealand, members of the public are encouraged to return unused medicines to their local pharmacy for disposal. Medicines should not be disposed of as part of normal household refuses because of the potential for misuse and because municipal waste disposal in landfills is not the disposal method of choice for many pharmaceutical types. Handling and disposal should comply with the guidelines in NZ Standard 4304:2002—Management of Healthcare Waste. Medicines should be disposed of by high temperature incineration, or by other means that render the substances non-hazardous to the environment. All areas in New Zealand have access to services for the collection and appropriate destruction of pharmaceutical waste (New Zealand Pharmacy Network 2009).

However, many of the reviewed studies noted absence of the functioning take-back programs in surveyed countries. For example, in Serbia, the government introduced legislation on pharmaceutical waste in 2010 which obliges pharmacies

to collect unwanted medication from citizens, but does not clearly identify the responsible of financing storage and destruction of the waste because both wholesalers and manufacturers avoid this obligation (Kusturica et al. 2012). This discrepancy between legislation and practice probably contributed to almost 90 % of Serbian public using household garbage as usual mean of disposal. In Ireland the public campaigns on proper medication disposal are intermittent and of limited impact, and even though public is advised to return medicines to the pharmacy for safe disposal pharmacies are under no obligation to accept returns and the costs of disposal (Vellinga et al. 2014). In Thailand, the community pharmacy association ran 'Return Your Unwanted Medicines to Pharmacy' program to promote a more appropriate disposal of unwanted medicines, but it was not a continuous program and, therefore, it was not strong enough to build up public awareness regarding this issue. In 2012, the Ministry of Public Health launched a project called 'Exchange Old Unused Medicines for Eggs' in order to estimate the extent and value of unused medicines. Unfortunately, this project lasted 1 week and the amount and value of returned medicines have not yet been revealed to public (Old Medicines for Eggs, 2012) (Arkaravichien et al. 2014). In Ghana, currently there are only two quasi-governmental hospitals and their subsidiary clinics which have a comprehensive drug return program called DUMP (Disposal of Unused Medicines Program). However, this program is not widely known as the results of the study indicated that more than 80 % of the respondents had never heard about any drug return program in the country (Sasu et al. 2012). No official guidelines for disposal of unused medications exist in Kuwait (Tong et al. 2011). Nevertheless, a pilot collection program was attempted in 200 households (Abahussain and Ball 2007), when the residents were given special bags to place unused medications, which were then to be collected by the municipality. Although more than a half of participants (54 %) thought this was appropriate way of collection and disposal, none of the households involved actually utilized it. In a subsequent study, researchers went to residents and spoke to citizens about the risk unused medications posed to humans and environment and only after this direct intervention unused medications were collected. In Kingdom of Saudi Arabia there is an act issued in 1999 by the Ministry of Health and another issued in 2001 by the Gulf Countries Council (GCC) regulating the management of medical waste including unwanted pharmaceutical compounds. However, these acts are not specific when they come to receiving and disposing returned medicines from the public. There is also no take-back program to accept unused prescribed drugs from public (Abdallah et al. 2014). In Qatar, there is currently no plan or system for the management of domestic medical waste, but there is a project under the leadership of Supreme Council of Health to manage this source of medical waste. No specific legislation on management of pharmaceutical waste or organized drug take back programs exists in Oman, Bangladesh, India and Nigeria (Abdo-Rabbo et al. 2009; Emdadul et al. 2012; Ugwuh 2009; Patil and Patil 2013).

4.3 Impact of Knowledge and Educational Campaigns on Disposal Practices of Unwanted Residential Medication

While it is clear that environmental awareness may impact the choice of what is considered the safest mean of disposal, actual behavior does not always equate the awareness. However, this gap between the possession of environmental knowledge and awareness, and displaying pro-environmental behavior is well known. For decades, researchers have sought to understand and map the factors that lead people to delay or to fail to move from environmental knowledge to pro-environmental behavior. An individual's perception of the extent to which his or hers actions can truly help to improve the environment and sense of personal responsibility can both contribute to people's behavior (Gifford and Nilsson 2014). In the last decade environmental psychology has made considerable progress in identifying central psycho-social determinants of people's intention to choose the pro-environmental behavioral option. Environmental behavior is a complex mixture of self-interest and pro-social motives and interplay of cognitive, emotional, and social factors (Bamberg and Moser 2007).

From relatively small number of reviewed studies, only two reported positive and strong association between knowledge and behavior regarding the disposal practices. A study carried out in California, USA, showed that less than half of the respondents are aware that medications have been found in treated wastewater and in surface waters, but respondents that are aware of the issue are less likely to use trash and toilet/sink for disposal and more likely to return drugs to the pharmacy (Kotchen et al. 2009). Similar pattern was observed in Sweden, where awareness of the effects of pharmaceuticals deposited in the environment was in accordance with both perceived and actual the best practices among general public (Persson et al. 2009).

No clear and definite connection between environmental awareness, what is perceived as the best option for disposal and actual disposal practice can be proved. As mentioned before, while environmental knowledge may lead to environmental awareness and concern, it does not necessarily lead to pro-environmental behavior. For example, Bound et al. reported finding no evidence of a link between risk perception and responsible disposal. Even though people with strong pro-environmental attitudes were found to be more likely to engage in pro-environmental behavior, the relationship between attitudes and actions proved to be weak (Bound et al. 2006). In Serbia, despite the fact that one-half of the population is aware of the harmful effects of medications on the environment, most of the population still disposes unused medications in a way that can have serious consequences on the environment (Kusturica et al. 2012). Similar situation was noted in Kuwait, where there is a large discrepancy between the habits and opinion regarding environmentally correct disposal of unused drugs (Abahussain and Ball 2007). The discrepancy between knowledge and behavior has been observed in UK, where even with the high level of recognition of the importance of proper disposal,

60 % of respondents disposed of these drugs via trash or sewer system (Bound et al. 2006). U.S. study reported that most respondents had believed that disposing medications in a secured lockbox located in a pharmacy or physician's office was the best method, yet less than 10 % used it as the actual mean of disposal. Law et al. observed lack of consistency and knowledge about disposal of unused medication despite respondents' high educational level and relationship with health care fields (2014). A gap in information and awareness of the problem of disposal of medicine seems to be present in the population overall, regardless of geographic region.

4.4 Future Perspectives and Possible Solution

It is important to emphasize that adequate legislation and functioning state run system are only the first necessary step in implementing proper disposal practices in the community. We strongly agree with the opinion of Wieczorkiewitcz et al. that pharmaceutical waste disposal costs should be funded by local authorities, health insurance companies, pharmaceutical industry or the government as the general population is not willing to carry the cost of unwanted residential medication disposal (Wieczorkiewicz et al. 2013). Most of the public consider pharmacies a suitable place for permanent drug drop boxes where residents can drop off unwanted, expired, or unused medicaments (Fenech et al. 2013; Law et al. 2014; Aditya 2013; Kotchen et al. 2009). In U.S. study, most believed that disposing medications in a secured lockbox located in a pharmacy or physician's office was the best method (65 %) while the least confidence (13 %) was put in a mail-back program (Wieczorkiewicz et al. 2013). Research conducted in Malta and Ireland showed that although less than 10 % of respondents return their unused pharmaceuticals to the pharmacy, most perceive it to be the best option. The main motivators for respondents that already dispose of their unused medications at pharmacies are safety and environmental health concerns (Fenech et al. 2013). If possible, pharmacies should be recompensed for providing the collecting service to accelerate the collection scheme implementation. Practice where pharmacies are considered producers and holders of pharmaceutical waste and therefore obliged to finance the service of collecting unused medicines from the public leads to numerous complicates and slows operation of medication collection scheme. This is the case in Croatia, were the amounts of collected medicines are below the European average per capita annually. According to the results of a Croatian study, functioning of the service is negatively influenced by the type of pharmacy ownership, distribution of pharmacies and lack of anonymity when disposing unused medicines. Furthermore, the type of ownership (wheatear it is private of sate-own) is associated with financial burden for pharmacies. Actually, in most European countries it is not common to find a model where pharmacies are financially responsible for the waste disposal; on the contrary, they are compensated for providing the service (Jonjic and Vitale 2014).

However, even where reverse distribution network is present, the actual rates of returning medication to collection sites are scarce. The most common reason for not returning medications to pharmacies or other collection sites was lack of information and awareness on the existence of available unwanted medication collection schemes in the community (Fenech et al. 2013; Iabu et al. 2013; Krupiene and Dvarioniene 2007, Sasu et al. 2012). Most of the people have never heard about such a possibility and do not know anything about the issue (Krupiene and Dvarioniene 2007). Iabu et al. reported that even though the respondents were medical students, 90 % were not aware of possibility of returning drugs to pharmacy (2013). Closing this gap in information is necessary. Pharmacists play the key role in reaching out and educating their patrons on proper medication disposal methods (Law et al. 2014; Wieczorkiewicz et al. 2013). Previous counseling about medication disposal positively influences beliefs concerning unused and expired medication disposal. Respondent who received advice on medication disposal were significantly more likely to believe that it is acceptable to return drugs to pharmacy or physician's office and to believe that it is not acceptable to store medication at home, or flush down the sink or drain (Seehusen and Edwards 2006). Vellinga et al. reported that out of people who in the past received advice on medication disposal, 75 % agreed that returning medication to collection sites was suitable and safe method of disposal, but only 19 % of participants have ever received instructions (2014). Following educational campaigns, majority of respondents are generally willing to change their current disposal practices (Abrous et al. 2010). Vellinga et al. (2014) reported that in Ireland, the major determinant for whether respondents had received information was level of education, which implies that even when education campaigns take place, certain sectors of society may be omitted.

In our opinion, targeted educational campaigns should be organized in the community, but advice on proper drug disposal should routinely follow drug dispensing. Educating general public about environmental issues is essential step in changing disposal practices, but it will not automatically result in more pro-environmental behavior. Key to translating knowledge into action is to make the action easy, use the familiar locations such as pharmacies as collection sites, and promote routinely and continuously the importance of proper medication disposal. We believe that with strict legal framework, well organized and implemented reverse distribution network, education and increase of environmental awareness the practice of medication disposal in the community can change for the better. However, we must consider that even after available take-back programs are implemented, it can take years of active educational campaigns before any significant results are achieved, so the action needs to be taken as soon as possible.

The review had some limitations that need to be mentioned. First of all, the search was limited to several search terms, and focused on peer reviewed literature published in English. The paucity of literature in this field of research combined with the variation in the techniques of data collection and reporting in the primary

studies which were included made it difficult to synthesize and draw conclusions. The use of non-random methods in participant recruitment in addition to not validating the data collection tools was common in many surveys and could potentially have an effect on the study outcomes. A final limitation is the use of self-report measures which are subject to recall bias, and often had unknown validity or reliability.

5 Conclusion

The present evidence gathered from a number of studies carried out in different geographical and demographical settings implies that general public still lacks knowledge regarding proper disposal of unused drugs and the environmental risk associated with inappropriate medication disposal. The current data suggests that improper disposal of pharmaceuticals is a global problem which plays a significant role in environmental contamination. Improper disposal is still prevalent among environmentally-aware people, which suggests that knowledge and awareness of environmental issues regarding medicines disposal only partially accounts for people's medicine disposal habits. While it is clear that environmental awareness may impact the choice of what is considered the safest mean of disposal, actual behavior does not always equate the awareness. Strict legal framework, well organized, cost-effective and easily accessible state-run disposal systems are necessary in order to enable the general public to reduce negative pharmaceutical impacts on the environment by returning unused pharmaceuticals to collection schemes for proper disposal.

6 Summary

The aim of this systematic review was to determine the practice of medication disposal around the world and get insight into possible link between environmental awareness and people's behavior regarding this issue. A literature search (2005–2015) was performed to identify reports with quantitative data on disposal practices published in peer-reviewed literature. The keywords "medication OR medicine OR drugs" AND "disposal OR unused OR wastage" and "household OR residential OR home" were used in searches involving following databases: PubMed, Web of Science, Google Scholar, Scopus and The Cochrane Library. The most common method for disposal of unused medications in households is disposal in the garbage (Kuwait, United Kingdom, Lithuania, Qatar, Serbia, Ghana, Bangladesh, Malta and Saudi Arabia). The practice of flushing drugs into the sewage system still takes place in New Zealand, USA and Bangladesh. Only in

Sweden and Germany, practice of returning drugs to pharmacy was practiced to a larger extent. Home backyard burning of expired and unused medication was commonly reported mean of disposal in Lithuania for respondents living in the countryside. The environmental impact of improper medication disposal is expected in countries with poorly functioning waste management schemes (Middle Eastern, Asian and African countries). Lack of the adequate information and clear instructions on proper manners of drug disposal was reported in many surveyed countries (USA, New Zealand, Bangladesh, Malta and Ireland). Clear and definite connection between knowledge about environmental detrimental effects of improper drug disposal and the preference towards disposal methods could not be established. Many respondents were generally concerned with issues of inadequate medicines discarding but the behavior regarding disposal of unused drugs often did not equate the awareness (Serbia, USA, Kuwait, Malta and UK). The current data emphasizes the global issue of improper medicine disposal, prevalent in environmentally-aware people. The present evidence gathered from a number of studies carried out in different geographical and demographical settings implies that general public still lacks knowledge regarding proper disposal of unused drugs and the environmental risk associated with inappropriate medication disposal. Strict legal framework, well organized, cost-effective and easily accessible state-run disposal systems are necessary in order to enable the general public to reduce negative pharmaceutical impacts on the environment by returning unused pharmaceuticals to collection schemes for proper disposal.

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Alkyl Mercury-Induced Toxicity: Multiple Mechanisms of Action

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Contents

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1 Preface

The alkyl mercury compounds methylmercury (MeHg) and ethylmercury (EtHg) have been shown to be toxic to humans and non-human animals as well (Driscoll et al. 2013). Both of those compounds have similar chemical properties, and both have been shown to disrupt the normal function of the CNS in a variety of animal species.

In mass poisonings from the consumption of MeHg-contaminated seafood in Japan (Kutsuna 1968) and MeHg-treated grain in Iraq (Bakir et al. 1973), frank developmental/birth effects and/or severe brain damage were observed in the children of some mothers who consumed large quantities of mercury-contaminated fish, bread, or grain products during pregnancy. In addition, a variety of neurologic effects were reported in both the 1968 and 1973 papers. Among these effects were deficits in cognitive and motor function. Some of the many specific effects observed were distal paresthesias, ataxia, unsteady gait, muscle weakness, impairment of the senses, irritability, memory loss, insomnia, and confusion. Similar neurologic disorders were seen in Iraq in 1956 and 1960, when food containing flour made from grain treated with EtHg *p*-toluene sulfonanilide was consumed (Bakir et al. 1973; Jalili and Abbasi 1961). In another reported incident (Zhang 1984), 41 individuals were poisoned by eating rice that had been treated with a grain disinfectant containing 2–2.5 % $EtHgCl_2$. Many of the symptoms and clinical signs were similar to those experienced in the ethyl- and methylmercury poisonings in Japan and Iraq. And while both of the alkyl compounds primarily effect the central nervous system, the rapid metabolism of ethylmercury to inorganic mercury may lead to kidney damage (Clarkson and Magos 2006), especially at higher amounts and with longer periods of exposure.

1.1 How Alkyl Mercury Compounds Get into the Food Web

Mercury enters the atmosphere from both natural sources (e.g., volcanos) and anthropogenic activities (e.g., burning of fossil fuels and industrial processes). As it gradually settles onto land and bodies of water, it is methylated by bacteria and other microorganisms to form MeHg. In aquatic and marine environments, the MeHg is biomagnified as it passes to larger and larger predatory organisms in the ecosystem. Top predatory fish typically have the greatest concentrations of MeHg in their muscle tissue.

The neurotoxic effects of alkyl mercury compounds appear to be similar across the vertebrate species studied. A primary source of exposure for humans and large marine animals is fish and shellfish. Many species of birds are piscivorous, so it logically follows that a number of bird species have been found to be negatively impacted by MeHg (Seewagen 2010). The effects reported in such species are primarily of neurologic origin and include ataxia, lethargy, reduced appetite and egg production, poor hatching success, and aberrant parental care. Non-avian

species that feed on fish are also exposed to MeHg, and would be expected to manifest symptoms and clinical signs of MeHg effect, just as with humans and birds. Elevated MeHg levels have been reported in aquatic mammals that feed largely on fish, including pilot whales (Weihe et al. 1996), elephant seals (Cossaboon et al. 2015) and mink and river otter (Yates et al. 2005). But since there is currently a relative paucity of literature on the effects of alkyl mercury compounds and their respective mechanisms of toxic action compared with the plethora of such studies regarding humans, the information presented in this paper focuses primarily on mechanisms of action on human cells and tissue cultures.

2 Introduction

The affinity of mercury for sulfur has been known since the days of alchemy (Hughes 1957), and the strong affinity of sulfhydryl (-SH) groups for mercury has often been considered to be involved in the mechanism of toxic action of mercury and its compounds. Clarkson et al. (2007) reported that the high mobility of methylmercury (MeHg) in the body is due to the formation of small molecular weight thiol complexes that are readily transported across cell membranes.

There are a number of mechanisms by which alkylmercury compounds cause toxic action in the body (Faustman et al. 2002). This paper represents a summary of some of the studies regarding these mechanisms of action in order to facilitate the understanding of the many varied effects of alkylmercurials in the human body. The data reveal that there are some similarities between the mechanisms of the two mono-alkyl mercury compounds: methylmercury (MeHg) and ethylmercury (EtHg).

Mercury in any form will bind with sulfhydryl (-SH) groups in thiols or proteins. Sulfur is present in the amino acids cystine, cysteine, and methionine, as well as the cysteine-derivative taurine, which is plentiful in the bile. Since receptors in/on cell membranes are all constructed from proteins, those receptors are susceptible to attack by various forms of mercury. The majority of protein transport systems in higher eukaryotes contain cysteine, and the presence of cysteine residues exposed towards the extracellular environment make them potential sites of interaction with mercurials (Pochini et al. 2013). When mercury binds to one of the amino acid residues in receptor proteins, it blocks or attenuates that protein molecule's range of availability for normal metabolic function. When the affected protein is normally used to transport a substance, such as calcium ion (Ca^{++}), across the membrane of a cell or sub-cellular organelle, the selective permeability of that membrane and the proper physiologic function of that cell or organelle may be compromised.

Overall, the data reveal that there are some remarkable similarities between the toxic mechanisms of the mono-alkyl mercury compounds MeHg and EtHg. However, due to differences in metabolic rates and the subsequent elimination half-lives, the methyl and ethyl forms of organic mercury are presented separately in this paper. The effects of MeHg are provided in Table 1. The effects of

Table 1 Some mechanisms of toxic action proposed for MeHg

Mechanism	Results	Reference(s)
Altered membrane permeability	MeHg attaches to cystine, enabling it to enter cells via a neutral amino acid transporter that also transports methionine into cells; also increases mitochondrial membrane permeability	Clarkson (1995); Clarkson et al. (2007); Pochini et al. (2013); Simmons-Willis et al. (2002)
Altered intracellular calcium homeostasis	Blocks cellular membrane Ca^{++} channels; causes release of mitochondrial Ca^{++} into the cytosol	Hare et al. (1993); Kang et al. (2006) Limke et al. (2004b); Marty and Atchison (1997); Minnema et al. (1987); Peng et al. (2002); Sirois and Atchison (2000); Szalai et al. (1999)
Oxidative stress/reactive oxygen species (ROS)	MeHg causes increase in ROS and subsequent depolarization of the mitochondrial membrane; decreases aconitase activity	Dreiem and Seegal (2007); Garg and Chang (2006); Myhre et al. (2003); Yin et al. (2007)
Effects on receptor binding/neurotransmitter release	Blocks GABA receptor sites; binds to NMDARs, inducing their expression; causes up-regulation of muscarinic ACh receptors	Basu et al. (2008); Coccini et al. (2000); Cooper et al. (2003); Fonfria et al. (2001); Ndountse and Chan (2008); Yuan and Atchison (2003)
Generation of arachidonic acid	Promotes the release of arachidonic acid, resulting in neurotoxicity	Shanker et al. (2002); Verity et al. (1994)
Effects on cycle/cell division	Causes arrest in the GM2/M-phase of the cell cycle through microtubule disruption	Burke et al. (2006); Castoldi et al. (2000); Gribble et al. (2005); Kim et al. (2007); Ou et al. (1999b); Rodier et al. (1984)
Effects on glutathione activity	Decreases glutathione activity, particularly in the cerebellum and mitochondria, thus providing less protection from oxidative stress due to MeHg	Carocci et al. (2014); Choi et al. (1996); Franco et al. (2006); Mori et al. (2007); Ndountse and Chan (2008)
Involvement with nitric oxide synthetase	MeHg causes an increase in nNOS protein, causing the overproduction of NO	Chuu et al. (2001); Shinyashiki et al. (1998)
Glutamate—glutamine uptake and homeostasis in glial cells	Disrupts glutamate homeostasis by disrupting glutamine/glutamate cycling; causes glutamate release from pre-synaptic neurons	Farina et al. (2003a, b); Manfroi et al. (2004); Yin et al. (2007)
Activation of calpain cascade leading to apoptosis	Cascade begins with increased intracellular Ca^{++} leading to microtubule degradation and cell death	Sakaue et al. (2005)

EtHg/thimerosal are provided in Table 2. Because of the controversy between the preservative thimerosal (CDC 2015), which is metabolized to ethyl mercury and thiosalicylate, a comparatively benign compound, there is much more published

Table 2 Some mechanisms of toxic action proposed for EtHg/thimerosal

Mechanism	Results	Reference(s)
Altered intracellular calcium homeostasis	Mobilization of intracellular Ca^{++}; causes release of mitochondrial Ca^{++} into the cytosol; attenuates any increase in internal calcium ion concentration	Elferink (1999); Machaty et al. (1999); Sayers et al. (1993); Tornquist et al. (1999); Zarini et al. (2006)
Oxidative stress/reactive oxygen species (ROS)	EtHg causes increase in ROS and depolarization of the mitochondrial membrane; inhibits mitochondrial respiration; increased formation of superoxide and hydrogen peroxide	Sharpe et al. (2012)
Generation of arachidonic acid (AA)	Promotes release of AA; prevents reacylation of AA	Chen et al. (2003); Stuning et al. (1988)
Effects on cell division	Thimerosal destroyed cell spindle, arresting embryonic development	Machaty et al. (1999)
Effects on glutathione activity	Inhibits glutathione-S-transferase T1 (GST T1); depletes GSH	Muller et al. (2001); Wu et al. (2008)
Involvement with nitric oxide synthetase	Causes an increase in NOS	Chen et al. (2003)
Effects glutamate transport	Disrupts glutamate homeostasis by disrupting glutamine/glutamate cycling; decreases GLT-1 levels; significantly inhibition of GLAST activity	Mutkus et al. (2005)

data regarding thimerosal than EtHg by itself. Consequently, most of the studies regarding the toxicity of EtHg consist of experiments using the vaccine preservative thimerosal, in which the effective portion is EtHg.

3 Methylmercury: Mechanisms of Toxic Action

Since Aschner and Aschner (1990) published that MeHg associates with thiol-containing amino acids due to the high affinity of the methylmercury cation ($MeHg^+$) for sulfhydryl groups (-SH), many studies have further explored the mechanism(s) by which MeHg exerts its toxic effects on mammalian cells.

When mercury binds to protein receptors, the effects that occur following, or as a result of, that binding are key to understanding the ultimate mechanism or mechanisms of toxic action of mercury. Since these effects occur at both the cellular and sub-cellular levels, in vitro and in situ studies are necessary to examine the chemical mechanisms occurring at these levels. Together, in vitro and in situ studies have provided the most valuable evidence and explanations of exactly why and how mercury and its organic compounds cause damage to nervous tissue.

A number of recent studies have examined the sub-cellular mechanism of the neurotoxicity of MeHg. Impaired calcium homeostasis (Dreiem and Seegal 2007; Sirois and Atchison 2000), oxidative stress (Garg and Chang 2006; Ou et al. 1999a; Yin et al. 2007), and the alteration of glutamate homeostasis (Farina et al. 2003a, b; Ou et al. 1999a; Yin et al. 2007) have all been suggested as possible mechanisms contributing to neurotoxicity. This paper will discuss these proposed mechanisms, in addition to a number of other mechanisms of action. Kang et al. (2006) reported that cell damage caused by MeHg may occur through more than one mechanism, the effects of which may be additive or synergistic. Ndountse and Chan (2008) also indicated the likelihood of more than one mechanism contributing to the expression of MeHg neurotoxicity. After reviewing the literature, we believe that there are indeed multiple mechanisms of action, which individually and collectively contribute to the adverse effects of MeHg on human health.

3.1 Altered Membrane Permeability

In a review of the mechanisms of mercury distribution in the body, Clarkson et al. (2007) described how the high mobility of MeHg in the body is due to the formation of small molecular weight thiol complexes that are readily transported across cell membranes. When MeHg attaches to the amino acid cysteine, the resulting structure mimics that of methionine, a large neutral amino acid (Clarkson 1992, 1995). This enables the MeHg-cysteine complex to enter cells on neutral amino acid transporters that also transport methionine into the cells. This is supported by the fact that the uptake of MeHg into the brain is inhibited by the presence of large, neutral amino acids such as leucine, methionine, and phenylalanine, and others (Clarkson 1995). Evidence presented by Clarkson et al. (2007) is also supported by the work of Simmons-Willis et al. (2002), who showed experimentally that the MeHg-L-cysteine complex is a substrate for human L-type large neutral amino acid transporters (LAT) LAT1 and LAT2.

Most MeHg in tissues is normally complexed with water-soluble sulfhydryl-containing molecules, primarily L-cysteine, GSH, hemoglobin, albumin, and other cysteine-containing polypeptides (Simmons-Willis et al. 2002). One possible explanation for the wide distribution of MeHg in the body is that an endogenously formed MeHg complex acts as a substrate for transporters that are also widely distributed throughout the body. Simmons-Willis et al. (2002) investigated the possibility that MeHg-L-cysteine, which is structurally similar to methionine, is a substrate for LAT. LAT1 and LAT2 are expressed in many tissues, including brain, and mediate the uptake of large neutral amino acids (Palacin et al. 1998; Verrey et al. 2000).

Using oocytes from the African clawed toad (*Xenopus laevis*), which expresses two of the L-type carriers expressed in humans [LAT1-4F2 heavy chain (LAT1-4F2hc) and LAT2-4F2hc], Simmons-Willis et al. (2002) injected oocytes with 50 nL of 110 mM stocks of amino acids. This resulted in intracellular concentrations of ~10 mM just prior to taking uptake measurements. In another

part of the study, healthy oocytes were loaded with either [^{3}H]methionine, and a 10:1 molar ratio of L-cysteine and [^{14}C]MeHg giving approximate intracellular concentrations of 1 and 0.1 mM (100 μM), respectively.

Control oocytes were able to accumulate methionine from the medium containing 100 μM tritiated methionine, due to the presence of endogenous amino acid transporters. But when oocytes were injected with cRNA for LAT1-4F2hc or LAT2-4F2hc, methionine uptake was greatly enhanced. Further, LAT1- and LAT2-expressing oocytes also showed higher uptake of [^{14}C] MeHg when applied as 100 μM MeHg-L-cysteine. Simmons-Willis et al. (2002) reported that the LAT1- and LAT2-stimulated uptake of MeHg from 100 μM MeHg-L-cysteine was significantly ($p < 0.05$) higher than from 100 μM methionine, indicating that the MeHg-L-cysteine complex may make an excellent substrate for these amino acid transporters. Those authors concluded that the LAT1 and LAT2 proteins may provide the route of MeHg entry into brain and other tissues and may account for the rapid and widespread tissue distribution of MeHg.

Pochini et al. (2013) provided supporting data by using proteoliposomes to investigate the effects of MeHg on the carnitine (OCTN2) transporter activity. Proteoliposomes have been shown to be an appropriate experimental model for investigating the interaction of transporters with chemical compounds, including MeHg (Oppedisano et al. 2010, 2011). Carnitine is an acyl carrier with respect to the mitochondrial membrane and stimulates fatty acid oxidation and synthesis in the mitochondria.

In their study, Pochini et al. (2013) added 10 μM [^{3}H]carnitine to proteoliposomes. The subsequent addition of 8 μM MeHg was found to strongly inhibit carnitine transport. Fifteen minutes after the application of the MeHg, the transport was inhibited by more than 50 %.

To test whether the cysteine (Cys) sulfhydryl groups could be involved in the observed inhibition of the carnitine transporter, Pochini et al. (2013) added 2 mM dithioerythritol (DTE) to the proteoliposomes after they were incubated with MeHg. DTE is a sulfur-containing sugar, known to be an excellent reducing agent. The addition of the DTE resulted in recovery of the transport. In contrast, DTE had virtually no effect when added to control proteoliposomes. These results indicated that the thiol groups of the protein were involved in the binding with MeHg. From their studies, Pochini et al. (2013) concluded that the interaction of mercury with transport systems essential in cell homeostasis seems to be a general mechanism of toxicity.

Garg and Chang (2006) examined the effects of MeHg concentrations from 0.5 to 40 μM on mitochondrial membrane potential in microglia. In this experiment, they observed significant depolarization of the mitochondrial membrane with increasing MeHg concentrations, but statistically significant ($p < 0.001$) only at concentrations of 20 μM or higher.

3.2 Altered Intracellular Calcium Homeostasis

In a review of possible mechanisms for the cytotoxic action of MeHg, Limke et al. (2004b) noted that intracellular calcium $[Ca^{++}]_i$ undergoes cyclic changes in concentration during normal neurologic function, and that a large concentration gradient of calcium ion concentration typically exists across the neuronal membrane. As a result, the sustained elevation of $[Ca^{++}]_i$ can be deleterious in two major ways: (1) a depletion of energy reserves resulting from excessive employment of ATP-dependent Ca^{++} pumps to restore resting membrane potential; and (2) the activation of catabolic functions, both of which can contribute to Ca^{++}-mediated cell death (Limke et al. 2004a, b).

Further, Peng et al. (2002) provided evidence that MeHg disrupts normal Ca^{++} channel functions, as seen in studies measuring the influx of radiolabeled calcium in synaptosomes (pinched-off nerve endings), ligand binding, and studies employing electrophysiological techniques.

One of the functions of the smooth endoplasmic reticulum (SER) is to serve as a storage site for $[Ca^{++}]_i$. As such, Ca^{++} in the cytosol must be actively transported against its concentration gradient into the SER, a process requiring ATP. Bearss et al. (2001) reported that the application of thapsigargin, an inhibitor of smooth endoplasmic reticulum Ca-ATPase activity, reduced the amplitude of MeHg-induced increase in $[Ca^{++}]_i$ by 30 % in rat cerebellar granule cells in primary culture.

Hare et al. (1993) used single NG108-15 cells preloaded with the fluorescent dye fura-2 to evaluate whether MeHg caused an increase in $[Ca^{++}]_i$. Whereas 0.5 μM MeHg had no effect, both 2 and 5 μM MeHg produced a biphasic increase in fluorescence. The initial increase in fluorescence was sustained, with the time to onset being concentration-dependent. The maximum increase, however, was not found to be dependent on the MeHg concentration. The initial phase was considered to likely be the result of an increase in Ca^{++} from both intra- and extra-cellular sources, since removal of Ca^{++} from the extracellular medium reduced, but did not eliminate, the increase. The time to the onset of the second phase was also concentration dependent. Thus, MeHg was found to alter the measured fura-2 fluorescence in the cells in both a concentration- and time-dependent manner. Hare et al. (1993) also concluded that the initial effect involved alterations in intracellular cation buffering, as well as an increase in the permeability of the plasma membrane to Ca^{++}.

Sirois and Atchison (2000) used whole-cell patch clamp techniques to investigate the ability of MeHg to block Ca^{++} channel currents in cultures of neonatal cerebellar granule cells taken from 7-day-old rat pups of either gender. This cell type was chosen for this study due to their diversity of Ca^{++} channels, as initially reported by Randall and Tsien (1995). To determine whether MeHg is specific for one or more of the known sub-types of Ca^{++} channel receptor, Sirois and Atchison (2000) used a number of putative Ca^{++} channel antagonists (ω-conotoxin GVIA,

ω-conotoxin MVIIC, ω-agatoxin IVA, calcicludine, and nimodipine), each with specific blocking characteristics.

To eliminate the possibility of mixing the release of intracellular calcium with the influx of extracellular calcium through calcium channels, Sirois and Atchison (2000) used the radiolabeled divalent barium ion (Ba^{++}) instead of Ca^{++} in the bathing medium. While Ba^{++} is not normally a constituent of extracellular fluid bathing the neurons, the gated calcium channels are nonetheless highly permeable to Ba^{++}, making Ba^{++} a logical surrogate for extracellular Ca^{++}. In this study, the authors found that acute exposure to sub-micromolar concentrations of MeHg can block Ba^{++} currents carried through multiple Ca^{++} channel subtypes. They further reported that the channel-blocking effect of MeHg was seen well within concentrations seen during episodes of MeHg intoxication. And while the role that MeHg-induced calcium block plays in MeHg neurotoxicity remains to be determined fully, the low concentration at which these effects were seen in this study makes it likely that the effects on Ca^{++} channels at least contribute to the pathological damage observed in MeHg poisoning (Sirois and Atchison 2000). These authors further postulated that the blockage of Ca^{++} channels could possibly contribute to ultimate neuronal death in the cerebellum through the disruption of neurotransmitter release, cell growth and differentiation, protein synthesis, maintenance of the membrane potential, and regulation of $[Ca^{++}]_i$.

Additional support for the ability of MeHg to block Ca^{++} channels comes from work in that same lab which found that MeHg blocked Ca^{++} currents, although not completely, in human embryonic kidney cells in culture (Peng et al. 2002).

Altering intracellular calcium levels in brain capillary endothelial cells has a direct effect on blood-brain barrier permeability and transport (Paemeleire et al. 1999). Thus, substances such as organic mercury compounds, which cause the release of intracellularly bound Ca^{++}, might not only have a direct effect on neuronal function, but may also increase the availability of mercury, and possibly other neurotoxicants, in the CNS.

Minnema et al. (1987) also found evidence of MeHg-induced release of mitochondrial calcium from rat brain synaptosomes. In a multi-faceted experiment, these researchers found that MeHg produced large effluxes of calcium from isolated mitochondria preloaded with radio-labeled calcium, but not from synaptosomes preloaded with radiolabeled calcium. They also found that 0.5–5.0 μm MeHg caused a concentration dependent increase in the spontaneous release of tritiated dopamine from striatal synaptosomes, gamma-amino butyric acid (GABA) from cerebellar cortical synaptosomes, and acetylcholine from hippocampal synaptosomes. These releases were determined to not be attributable to a MeHg-increase in calcium permeability of the synaptosomal membrane, since these increases persisted in the absence of extrasynaptosomal calcium (Minnema et al. 1987).

Some of the effects of MeHg intoxication involve the cerebellum, and some of those cerebellar effects are due to the preferential targeting of MeHg. At low concentrations, MeHg has been shown to disrupt the ability of rat cerebellar granule cells in primary culture to maintain the intracellular calcium concentration ($[Ca^{++}]_i$). The mitochondria in cerebellar granule cells can store and release large amounts

of Ca^{++} (Budd and Nicholls 1996). One avenue of Ca^{++} release is through the mitochondrial permeability transition pore (MTP) (Bernardi et al. 1992; Bernardi and Petronilli 1996). Szalai et al. (1999) reported that, at the beginning of the apoptotic process, apoptotic stimuli induce a switch in mitochondrial calcium signaling by facilitation of a Ca^{++}-induced opening of the MTP. Thus, signals evoked by large Ca^{++} pulses or IP_3-mediated spikes in cytosolic Ca^{++} trigger mitochondrial permeability. Limke and Atchison (2002) demonstrated that MTP appears to play a significant role in the cellular effects following acute exposure of cerebellar granule neurons to MeHg.

Marty and Atchison (1997) had previously demonstrated that low (0.2–2.0 μM) concentrations of MeHg disrupt the ability of rat cerebellar granule cells to maintain $[Ca^{++}]_i$, resulting in a biphasic increase in $[Ca^{++}]_i$. Further, the initial phase involved release of intracellular Ca^{++} into the cytosol, followed by a secondary influx of extracellular Ca^{++}. It had also been previously demonstrated that the opening of the MTP could be inhibited by the immunosuppressant cyclosporine A (CsA) (Bernardi et al. 1993). Limke and Atchison (2002) found that exposure to CsA for 10 min prior to the addition of MeHg delayed the time-to-onset of both the first- and second-phase elevations of intracellular Ca^{++} in a CsA concentration-dependent manner. The delay in both phases suggested to the authors that the two phases of increased intracellular Ca^{++} were interrelated, with the first phase causing or contributing in some way to the second phase. Limke and Atchison (2002) thus felt that the induction of the MTP may link the first and second $[Ca^{++}]_i$ phases.

Kang et al. (2006) used both canine kidney and human neuroblastoma cells to further investigate the mechanism by which MeHg exerts its cytotoxicity. These researchers found that MeHg-induced toxicity was linked to the elevation of $[Ca^{++}]_i$ through the activation of phosphatidylcholine-specific phospholipase C (PC-PLC),

To first study the change in $[Ca^{++}]_i$, Kang et al. (2006) loaded the calcium binding dye fura-2 into Marbin-Darby Canine Kidney (MDCK) cells. MeHg was found to increase $[Ca^{++}]_I$ in a bimodal manner. Whereas the first phase sharply peaked by ~4-fold at approximately 80 s, the second phase was only gradually increased $[Ca^{++}]_I$ by ~2-fold at 400 s. In order to determine whether this increase came from extracellular calcium or intracellular calcium stores, $[Ca^{++}]_i$ was measured in the absence of extracellular Ca^{++}. To accomplish this, the calcium binding agent ethylene glycol tetraacetic acid (EGTA) was added to the extracellular medium. In the presence of EGTA, the MeHg-induced increase in $[Ca^{++}]_i$ was decreased by ~65 % in the first phase and ~30 % in the second phase, suggesting that the MeHg-induced increase in $[Ca^{++}]_i$ could come from both intracellular and extracellular Ca^{++} sources. One source is the mobilization of Ca^{++} from $[Ca^{++}]_i$ pools, and the other is through an increase in the permeability of the plasma membrane, as previously suggested by Atchison and Hare (1994).

Kang et al. (2006) also found that the antiviral, anti-tumoural xanthate D609, a competitive inhibitor of PC-PLC, almost completely abolished blocked MeHg-induced cytotoxicity in SH5YSY human neuroblastoma cells in a dose-dependent manner in both of the biphasic phases of $[Ca^{++}]_i$ increase, providing strong evidence of the role of PC-PLC as a critical pathway in mediating the MeHg-induced toxicity.

To investigate whether mitochondrial dysfunction is involved in MeHg-induced neurotoxicity, Yin et al. (2007) exposed astrocytes to concentrations of 1, 5, or 10 μM MeHg for 1 or 6 h. Mitochondrial membrane potential was measured by quantification of TMRE fluorescence. The mitochondrial membrane potential was significantly ($p < 0.001$) reduced both at 1 h and 6 h measurements at all three MeHg concentrations.

Garg and Chang examined the effects of MeHg concentrations from 0.5 to 40 μM on mitochondrial membrane potential in microglia. In this experiment, they observed depolarization of the mitochondrial membrane with increasing MeHg concentrations, but the effect was significant ($p < 0.001$) only at concentrations of 20 μM or higher.

3.3 Oxidative Stress/Reactive Oxygen Species (ROS)

One demonstrated mechanism of MeHg toxicity is the generation of reactive oxygen species (ROS), which can lead to neuronal cell death. An excessive presence of ROS can trigger cytotoxicity through a mechanism involving the endoplasmic reticulum (ER) (Makino et al. 2014). One function of the ER is to synthesize, fold, and process secretory and transmembrane proteins (Oslowski and Urano 2011). Proteins enter the ER as unfolded polypeptide chains, which fold and mature in the lumen of the ER (Ron and Walter 2007). When unfolded proteins accumulate in the lumen of the ER, a condition called ER stress can occur. This ER stress is created due to an imbalance between the load of unfolded proteins entering the ER and the capacity of the cellular machinery to handle this load (Ron and Walter 2007).

Makino et al. (2014) investigated the means by which methylmercury interacts with an important ER enzyme, protein disulfide isomerase [PDI]. PDI is responsible for catalyzing the formation of disulfide bonds of maturing proteins in the ER. Any agent that interferes with the action of PDI can cause the accumulation of newly synthesized unfolded proteins in the ER, leading to ER stress. Makino and his fellow investigators hypothesized that methylmercury would affect PDI in the ER by covalently bonding with the enzyme and reducing its ability to act. They incubated human neuroblastoma SH-SY5Y cells with various concentrations of MeHg. The effect of MeHg on the cells was measured by an MTT assay [3-(4,5-dimethylthiazol-2-thiazolyl)-2,5diphenyl-tetrazolium bromide (MTT) assay]. The MTT is a tetrazolium reduction coloration assay which examines the ability of the treated cells to metabolize MTT and is used as a measure of their viability. Through this assay, Makino et al. (2014) found a dose-dependent effect of MeHg on the viability of the cells, especially at MeHg concentrations >4 μM MeHg.

To test whether the decrease in viability was tied to ER stress, levels of two indicators of ER stress, CHOP (transcription factor C/EBP homologous protein) and BiP (immunoglobulin binding protein) were measured. These two ER stress indicators were elevated by the presence of MeHg. Matrix-assisted laser desorption/

ionization—time-of-flight (MALDI-TOF)/MS assays were also performed to specifically show that MeHg modifies PDI covalently at Cys 383 or 386 via bonding with thiol regions. They also looked at the effects of MeHg on the activity of PDI and found that it causes decreases in this enzyme's activity. Thus, Makino et al. (2014) proposed that one possible mechanism for neuronal cell death caused by MeHg is the binding of Cys residues of the endoplasmic reticulum enzyme, PDI. This MeHg interference with the action of PDI could lead to ER stress (specifically through a build-up of unfolded protein residues), which if not corrected by the machinery of the cell's unfolded protein response (UPR) could lead to cell death.

Limke et al. (2004a) examined the role of muscarinic receptors in MeHg-induced dysregulation in rat cerebellar granule cells in vitro through the use of fura-2 single-cell microfluorimetry. When atropine, a non-specific muscarine receptor antagonist, was added to the preparation, the onset of MeHg-induced Ca^{++} release into the cytosol was significantly delayed. In addition, depletion of smooth endoplasmic reticulum (SER) Ca^{++} with thapsigargin or down-regulation of muscarinic receptors and inositol-1,3,4-triphosphate (IP_3) receptors with bethanechol caused similar reductions in the amplitude of the MeHg-induced Ca^{++} increase. Collectively, these suggest that MeHg interacts with muscarinic receptors to cause Ca^{++} release from the SER through activation of IP_3 receptors.

Limke et al. (2004a) concluded that the results of their experiments suggested that interactions of MeHg with muscarinic receptors and resultant perturbation of Ca^{++} regulation within the SER may contribute to the selective vulnerability of cerebellar granule cells. Further, the importance of the SER as a MeHg target may lie in the effect of the released Ca^{++} on nearby mitochondria, and the localization of specific muscarinic receptor subtypes may contribute to the regional selectivity of MeHg toxicity within the CNS. These conclusions are supported by previous work in the same laboratory (Limke et al. 2003), which suggested that the SER contributes Ca^{++} to the observed mitochondrial dysregulation and subsequent neuronal death via an MTP-dependent pathway.

Active transport processes essential to the maintenance of the neuronal resting membrane potential require a great amount of the energy-carrying molecule adenosine triphosphate (ATP), which is produced in the mitochondria within the neurons through the oxidative metabolism of glucose. The effect of MeHg on mitochondrial metabolic function was examined by Dreiem and Seegal (2007) using rat striatal synaptosomes. Specifically, the study was designed to determine whether MeHg-induced elevations in reactive oxygen species (ROS) or alterations in intracellular calcium were the cause of compromised mitochondrial function. The formation of ROS was assessed through the use of dichloro-dihydro-fluorescein diacetate (DCFH-DA), a non-fluorescent, cell-permeable compound to form 2′7′-dichlorolfluorescein (DCF) (Myhre et al. 2003). Mitochondrial metabolic function was assessed by the ability to convert the dye methyl-thiazoletetrazolium to formazan. The authors reported that MeHg increased ROS levels, decreased mitochondrial function, and caused an increase in both cytosolic and mitochondrial

calcium levels in the synaptosomes. When the synaptosomes-MeHg preparation was co-incubated with Trolox, an antioxidant, the MeHg-induced ROS level was significantly reduced; however, the Trolox failed to restore mitochondrial function. The elevation in calcium levels was found to be independent of extra-synaptosomal calcium. The authors concluded that the MeHg-induced mitochondrial dysfunction is not the result of increased ROS levels, but instead the ROS increase is a secondary event in MeHg toxicity. Further, they suggested that MeHg-induced elevations in mitochondrial calcium are responsible for the mitochondrial damage caused by MeHg (Dreiem and Seegal 2007).

Garg and Chang (2006) examined the effects of MeHg at various concentrations on the murine N9 microglial cell line. They treated microglial cells with various concentrations of MeHg for 30 or 60 min and found that ROS increased in a time- and dose-dependent manner. At concentrations of 20 μM or above, the increase in ROS was statistically significant ($p < 0.001$). It had previously been shown that the enzyme aconitase is a sensitive intracellular target for oxidative stress (Gardner et al. 1994). Aconitase is an enzyme catalyzing the conversion of citrate to isocitrate in the tricarboxylic acid (or citric acid) cycle, and is thus crucial to the production of the energy-carrying molecule adenosine triphosphate (ATP) from glucose. Garg and Chang (2006) thus performed experiments to study the effects of MeHg on aconitase at concentrations of 0, 5, 10, or 20 μM MeHg. Aconitase activity was found to be impaired by MeHg, with activity dropping by 14 % at 5 μM, 53 % at 10 μM, and 90 % at a MeHg concentration of 20 μM. All decreases were statistically significant ($p < 0.01$ at 5 μM and $p < 0.001$ at 10 μM and 20 μM concentrations).

It had been previously reported (Eskes et al. 2002) that MeHg also decreased the production of the cytokine interleukin-6 (IL-6) in microglia. Garg and Chang also conducted experiments to study whether IL-6 production was affected in cultured N9 microglia. These cells were treated with various concentrations of MeHg for 8 h before measuring the production of IL-6 using ELISA. At concentrations of 10 μM, MeHg caused a significant increase in IL-6 production, despite the fact that this concentration greatly inhibited protein synthesis. The authors noted, however, that the role(s) of IL-6 in neurotoxicity is still unknown. Overall, Garg and Chang (2006) concluded that their study demonstrated that exposure to MeHg caused microglial cellular oxidative stress as determined by ROS generation, in addition to changes in mitochondrial membrane potential and aconitase activity.

Yin et al. (2007) studied the ability of MeHg to induce oxidative stress in primary astrocytes by measuring the levels of F_2-isoprostanes (F_2-Iso-Ps), a lipid peroxidation biomarker of oxidative injury. They found that 1 or 6 h of exposure of primary astrocytes to concentrations of 5 or 10 μM MeHg resulted in significant ($p < 0.05$) increases in F_2-Iso-Ps levels. The highest increases were observed at a concentration of 5 μM for 6 h, suggesting a maximal effect at this concentration. A MeHg concentration of 1 μM did not cause a significant biomarker increase, with F_2-Iso-Ps levels indistinguishable from controls.

3.4 *Effects on Receptor Binding/Neurotransmitter Release*

The muscarinic ACh (mACh) receptor has five different isotopes that regulate a diverse number of motor, sensory, and cognitive neurobehaviors (Basu et al. 2008). And while it is recognized that MeHg can affect the general population of mACh receptors and alter receptor protein levels, relatively little is known about the interaction of MeHg with specific isoforms of that receptor.

Therefore, Basu et al. (2008) investigated the effects of MeHg on muscarinic cholinergic receptor subtypes M1 and M2 using a combination of in vitro competitive binding assays and examination of tissues from MeHg exposed mink. In this study, juvenile male mink (9 per treatment group) were fed diets containing MeHg concentrations of 0, 0.1, 0.5, 1, or 2 μg/g (ppm) diet for 89 days. Animals were then sacrificed and the entire brain was extracted from each skull, after which the occipital cortex and brain stem were dissected from the right hemisphere. Pirenzepine, a selective anti-muscarinic agent, was used in tritiated form to block M1 muscarinic ACh receptors, and [^{3}H]-AFDX-384 was used as an M2 blocker.

In the in vitro tests, MeHg inhibited the binding of radioligands to the M1 and M2 receptors in the occipital cortex and brain stem of the mink. Whereas MeHg inhibition of [^{3}H]-pirenzepine binding to M1 receptors was observed in both the cortex and brain stem, the effect was more pronounced in the cortex. Similarly, [^{3}H]-pirenzepine in vitro binding results showed that MeHg was more potent at inhibiting ligand binding in the occipital cortex than in the brain stem (Basu et al. 2008).

Conversely, in vivo exposure of the mink to dietary MeHg resulted in greater binding of radioligands to both M1 and M2 receptors (Basu et al. 2008). While an exposure-dependent increase in [^{3}H]-pirenzepine binding was measured in both the occipital cortex and brain stem, only the increase in the cortex was statistically significant ($p < 0.01$, vs. $p = 0.2$ in the brain stem). In the case of [^{3}H]-AFDX-384, a MeHg-dependent increase in binding was observed in the occipital cortex of mink exposed to concentrations of 0.5, 1, and 2 ppm MeHg ($p < 0.01$), but the increase in binding in the brain stem was non-significant ($p = 0.1$). The authors explained this difference in direction of binding change between in vitro and in vivo studies as being logical. They pointed out that previous neuropharmacologic studies have established that exposure of animals to the muscarinic agonist atropine results in the up-regulation of receptor protein (Wall et al. 1992). The relevance to the Basu et al. (2008) study is straight-forward, as the receptor binding inhibition outside of a whole-body situation could not result in a compensatory increase in protein necessary to make new receptors. However, the response to blockage of receptors in vivo over a period of time would be the compensatory up-regulation of receptors.

In another study, adult female Sprague-Dawley rats were provided MeHg in drinking water at nominal concentrations of 0, 2.5, or 10 μg/L for 16 consecutive days (Coccini et al. 2000). Water consumption and body weights were obtained daily and used to determine daily intakes of 0.5 or 2.0 mg MeHg/kg/day for the 2.5 and 10 μg/L concentrations, respectively. The rats were decapitated either

immediately following the dosing period or 14 days after the cessation of MeHg administration. The cerebral cortex, cerebellum, and hippocampus were dissected out and examined for muscarinic receptors radiolabeled with [^{3}H]quinuclidinyl benzilate. The authors found evidence of up-regulation of muscarinic ACh receptors. The density of these receptors was increased in only the cerebellum and hippocampus, but receptor affinity remained unaltered in all three brain areas. While an increase in muscarinic ACh receptors was not initially seen in the cerebral tissue, it was demonstrable in the cerebrum of animals examined two weeks after the termination of treatment.

Coccini et al. (2000) concluded that prolonged ingestion of low doses of MeHg by rats causes subtle molecular changes with adaptive imbalance of muscarinic ACh receptors in the hippocampus and cerebellum. These authors also noted that because cholinergic systems play an important role in learning and memory, as reported by Mash et al. (1985), the increased ACh receptor density caused by MeHg may be a deleterious process.

Yuan and Atchison (2003) conducted experiments using whole-cell recording techniques to examine why cerebellar granule cells are much more sensitive to MeHg than are their neighboring cerebellar Purkinje cells. A specific area of interest was whether the expression of phenotypically different $GABA_A$ receptor alpha subunits plays a role in this differential sensitivity. The premise was that if so, the responses of $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) in Purkinje and granule cells should differ in their response to MeHg. Despite the fact that a range of MeHg bath concentrations was used, the pattern of response was similar. Biphasic changes were observed in frequency and amplitude of both spontaneous IPSCs and miniature IPSCs recorded from Purkinje and granule cells in a concentration and time-dependent fashion. However, the magnitude of the changes in frequency or amplitude of postsynaptic current was independent of the concentration of MeHg, suggesting that either a fairly constant series of events is initiated once an effective MeHg concentration is achieved, or that the concentrations tested were all at the high end of the concentration-response relationship. To use lower MeHg concentrations, however, the authors felt would compromise the high quality of the continuous whole-cell recordings due to the concentration-dependent latent period preceding the time of onset of the response.

While thus unable to prove their hypothesis, Yuan and Atchison (2003) did establish that MeHg acts at both pre- and post-synaptic sites to alter $GABA_A$ receptor-mediated inhibitory synaptic transmission. And while the general patterns of effects on the two cell types were similar, $GABA_A$ receptors in granule cells did appear to be more sensitive to blockage by MeHg than are those in Purkinje cells, as spontaneous GABAergic currents in granule cells were blocked at an earlier time than were those in Purkinje cells.

To determine whether MeHg interacts specifically with the $GABA_A$ receptor, Fonfria et al. (2001) used intact mouse cerebellar granule cells as an in vitro model of neuronal selectivity of that organomercurial. In this study, the authors investigated whether MeHg had an effect on granular cells pretreated for 30 min. with the radioligand [^{3}H]flunitrazepam, and found that binding was increased in a

dose-dependent fashion. They further found that this increase was completely blocked by bicuculline and picrotoxinin, both $GABA_A$ receptor antagonists, and by the organochlorine pesticide gamma-endosulfan as well. It was also found that the increase in [^{3}H]flunitrazepam binding in the presence of MeHg was independent of intracellular events, such as $[Ca^{++}]_i$, kinase activation/inactivation, or antioxidant conditions. Fonfria et al. (2001) concluded that MeHg interacts with the $GABA_A$ receptor by the way of alkylation of SH groups of cysteinyl residues found in $GABA_A$ receptor subunit sequences.

The effects of 40 μM, 400 μM, or 4 mM MeHg on the dopaminergic system of the rat striatum in conscious, freely moving animals were studied by Faro et al. (2000). All doses increased dopamine (DA) release (907 ± 31 %, 2324 ± 156 %, and 9032 ± 70 %, for low, middle, and high dose concentrations, respectively). High-dose exposure also caused significant decreases in extracellular levels of the DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (AV). The authors attributed these effects to the MeHg-stimulated DA release, decreased DA intra-neuronal degradation, or both.

Gao et al. (2008) investigated the effects of MeHg on postnatal neurobehavioral development in mice. Mice from the MeHg treatment groups were given intraperitoneal injections of 0.1, 1, or 3 mg/kg MeHg chloride from postnatal days 15 through 17, while control mice were injected with sterile saline. On postnatal day 45, the mice were tested using the Morris water maze to evaluate spatial learning and memory. Significant differences were seen between 1 mg/kg and 3 mg/kg groups and controls with regard to two measured parameters. Twenty-four hours after the final tests, three mice from each group were sacrificed and the hippocampus removed. Increases in NR1, NR2A, and NR2B subunits of the *N*-methyl-D-aspartate (NMDA) dopamine receptor were expressed in the hippocampus, relative to controls. Expressed as a percentage of control values, subunit NR1 (NMDAR1) was increased by 10 % in the 0.1 mg/kg group, 378 % in the 1 mg/kg group, and 314 % in the 3 mg/kg group mice. NR2A expression was increased by 21 % in 0.1 mg/kg mice, 256 % in 1 mg/kg mice, and 670 % in 3 mg/kg mice, while NR2B expression was enhanced by 59 %, 168 %, and 252 % in the 0.1 mg/kg, 1 mg/kg, and 3 mg/kg groups, respectively. Gao et al. (2008) concluded that the MeHg-induced subtle, but persistent, learning deficits and neurobehavioral abnormalities seen in the mice might be ascribed to alteration of the gene expression of specific NMDA receptor subunits in the hippocampus.

To investigate the role of the dopamine transporter in MeHg-induced dopamine release, Faro et al. (2002) administered adult female Sprague-Dawley rats MeHg dissolved in perfusion fluid and applied locally into the striatum via a dialysis probe. Intrastriatal infusion of 400 μM MeHg increased the extracellular dopamine levels to 1941 % of baseline levels (±199 %). The MeHg-induced release of dopamine was not attenuated in the total absence of calcium in the bathing Ringer's solution, nor was it attenuated after intraperitoneal (i.p.) pre-treatment with reserpine (a depletor of catecholamine stores in brain tissue) or the sodium channel blocker tetrodotoxin (TTX), suggesting that the dopamine release was independent of calcium and vesicular stores, as well as not affected by the blockade of

voltage-sensitive sodium channels. Following infusion of KCl (75 mM) through the dialysis probe, Faro et al. (2002) found that MeHg caused a decrease in the KCl-evoked release of dopamine. The authors concluded that collectively, their experiments suggest that MeHg induces the release of dopamine via a transport-dependent mechanism. The MeHg-induced release of dopamine was also independent of calcium and vesicular stores.

It has been known for some time that NMDA receptors (NMDARs) are widely distributed in the mammalian brain and spinal cord, with particularly high densities in the hippocampus and cerebral cortex. Over-activation or long term stimulation can damage and eventually kill target neurons via a process known as excitotoxicity (Cooper et al. 2003). Based on this knowledge, Ndountse and Chan (2008) hypothesized that MeHg binds to NMDARs and induces their expression, ultimately leading to cell death. They tested this hypothesis by investigating the effects of MeHg on the NMDA component of the excitatory neurotransmitter system. Specifically, they investigated the functional roles of NMDARs on the induction of cell death in the SH-SY 5Y neuroblastoma cell line after exposure to MeHg. The stated objective of their study was to investigate the effects of MeHg on the concentration of NMDARs in the membrane of neuronal cells and to explore the mechanism of toxicity involved.

In the Ndountse and Chan (2008) study, binding to the NMDAR was measured using the tritiated antagonist [^{3}H]-MK801. To measure the role of NMDA on the cultured neuroblastoma cells following MeHg application, the NMDAR antagonists MK801 (a.k.a. Dizocipine) and Memantine were used. Specific binding to the NMDARs was defined as the difference in [^{3}H]MK801 bound in the presence and absence of unlabeled MK801 (100 μm). Cytotoxicity was determined using the level of lactate dehydrogenase (LDH) in the supernatant released from the cells. MeHg treatment levels ranged from 0.1 to 5 μM.

Ndountse and Chan found that the cytotoxic effects of MeHg in human SH-SY 5Y neuroblastoma cultured cells were both time- and concentration-dependent. LDH increases of 20 %, 100 %, and 170 % were observed after 4 h of incubation with 1, 2.5, and 5 μM MeHg, respectively. After 24 h of treatment with 0.25, 0.5, 1, or 2.5 μM MeHg, LDH release was increased by 50 %, 120 %, 150 %, and 190 %, respectively, compared to control values.

Both of the NMDAR antagonists (100 μM) were found to significantly ($p < 0.05$) inhibit MeHg-induced neurotoxicity (72 % with MK801 and 70 % with Memantine). This finding was reported to suggest that the toxicity caused by MeHg was NMDA-dependent. To test for the involvement of Ca^{++} in the MeHg-induced neurotoxicity, Ndountse and Chan (2008) used the intracellular Ca^{++} chelator BAPTA-AM. They found that the inhibitory effect of BAPTA-AM on cytotoxicity was around 30 %, but not complete. The conclusion of Ndountse and Chan (2008) was that their study demonstrates that MeHg binds and increases NMDARs and induces apoptosis and necrotic cell death on human SH-AY 5Y neuroblastoma cells.

3.5 Effects Involving Arachidonic Acid

Arachidonic acid (AA) is an important component of the phospholipid component of mammalian cell membranes (Stuning et al. 1988). Cultured cerebellar granule cells (Verity et al. 1994) and astrocytes (Shanker et al. 2002) have been used to demonstrate the release of AA in the presence of MeHg. Verity et al. (1994) pre-labeled primary cerebellar granule cultures with tritiated ([^{3}H]) AA and exposed them to MeHg concentrations of 10–20 μM. The result was that MeHg induced [^{3}H]AA release in both a concentration-dependent and time-dependent fashion. Verity et al. (1994) also looked at the role of phospholipase A_2 (PLA_2) in MeHg-induced cytotoxicity, Cytosolic PLA_2 ($cPLA_2$) is important in the maintenance of membrane phospholipids, catalyzing the production of arachidonic acid and affecting its release. They found that PLA_2 activation preceded cytotoxicity in the cerebellar granule cells at lower, but not high, MeHg concentrations. Further, when the anti-malarial enzyme inhibitor mepacrine (100 μM) was present in the culture to inhibit MeHg-induced activation of PLA_2, the cytotoxicity was not altered. This could mean that either PLA_2 activation was not necessary for MeHg-induced cytotoxicity or that mepacrine failed to inhibit MeHg-induced PLA_2 activation.

Shanker et al. (2002) conducted a study designed to determine the effects of MeHg on the regulatory expression and activity of astrocytic $cPLA_2$ and AA release and look for a unifying target for MeHg-induced neurotoxicity. The astrocytes were obtained from cerebral cortices from 1-day-old Sprague-Dawley rat pups. The effect of MeHg on the activation of $cPLA_2$ was measured by the lease of [^{3}H]AA from astrocytes over a period of 120 min. Shanker et al. (2002) found a sustained increase in the rate of [^{3}H]AA release in astrocytes treated with 2.5 or 5.0 μM MeHg. This release was statistically significant ($p < 0.05$) at 10, 30, 60, and 120 min in astrocytes treated with 5.0 μM MeHg, but only at 120 min in cultured astrocytes treated with 2.5 μM MeHg. This was consistent with the time- and concentration-dependent finding of Verity et al. (1994). To test for the involvement of $cPLA_2$ in the observed [^{3}H]AA release following MeHg treatment, Shanker et al. (2002) used the specific $cPLA_2$ inhibitor $AACOCF_3$. They found that pre-treatment of astrocytes for 3 h with 10 μM AACOCF3 reduced the release of [^{3}H]AA following 5 μM MeHg to control levels ("statistically indistinguishable from control astrocytes"). The authors concluded that their results support the notion that cPLA2 stimulated hydrolysis and release of AA play a critical role in MeHg-induced neurotoxicity.

3.6 Effects on Cell Cycle/Cell Division

MeHg has long been known to interfere with the production of new neurons by adversely impacting the biological process of cell division (Rodier et al. 1984). This has been substantiated by a number of studies, including those reviewed next.

Castoldi et al. (2000) exposed in vitro cultures of rat cerebellar granule cells to MeHg concentrations of 0.5–1 μM or 5–10 μM. One hour of exposure to the higher concentration resulted in impairment of mitochondrial function and plasma membrane lysis, resulting in cell death. While the lower (0.5–1 μM) concentrations did not compromise cell viability or mitochondrial function at early time points, neuronal network fragmentation and depolymerization of microtubules were observed within 1.5 h. This damage continued to progress over time, and complete dissolution of microtubules and neuronal processes was seen after 18 h. The authors postulated that cytoskeletal breakdown and deprivation of neurotrophic support may play a role in delayed toxicity following MeHg exposure. Similarly, Miura et al. (1999) studied the relationship between changes in the cell cycle and the induction of apoptosis caused by MeHg in cultured mammalian cells, and reported that GM2/M-phase arrest through the disruption of microtubules is an important event in the development of apoptosis by MeHg. Consistent with altered mitotic division, Bahia et al. (1999) observed a reduction in the frequency of mitotic divisions following in vitro exposure of human lymphoblastoid (TK6) cells to MeHg.

While investigating the possibility that intracellular glutathione (GSH) synthesis may determine sensitivity to MeHg exposure, Ou et al. (1999a) found that while oxidative stress may mediate aspects of MeHg toxicity, disruption of GSH homeostasis alone is not responsible for the sensitivity of embryonic CNS cells to MeHg. In a separate study, Ou et al. (1999b) reported that the activation of cell cycle regulatory genes may be one mechanism by which MeHg interferes with the cell cycle in both adult and developing organisms.

Kim et al. (2007) used SH-SY5Y neuroblastoma cells to investigate whether or not MeHg alters the activity of regulatory proteins involved in the cell cycle. All-*trans*-retinoic acid (ATRA) was used in this study to induce differentiation of the neuroblastoma cells. The existence of retinoid receptors and related cytoplasmic binding proteins has previously been demonstrated in mammalian models (Maden et al. 1990; Ruberte et al. 1993); and it has been suggested that retinoids have a fundamental morphological action in the mammalian nervous system (Perez-Castro et al. 1989; Maden and Holder 1991). ATRA has been associated with several fundamental aspects of CNS development, including axonal growth (through modulation of nerve growth factor, or NGF), migration of elements of the neural crest (Perez-Castro et al. 1989; Maden and Holder 1991, 1992), and specifying the rostrocaudal position of the forebrain, midbrain, hindbrain, and spinal cord in the developing CNS (Maden and Holder 1992). Two days after ATRA-stimulated differentiation of neuroblastoma cells, Kim et al. (2007) performed cell cycle analysis using flow cytometry. Those researchers reported that MeHg treatment caused a significant change ($p < 0.05$) in the SH-SY5Y cell cycle. The G_1/G_0 portion of interphase was reduced in duration, and arrest of the S phase was reported.

Gribble et al. (2005) examined the role of p53, a major regulator of G_1 and G_2 stages of the cell cycle, in MeHg toxicity using isolated gestation day 14 (GD 14) mouse fibroblasts. In this study, mice heterozygous for the wild-type and null p53

allele were impregnated. At GD14, the pregnant mice were euthanized and gravid uteri were removed. Single cell fibroblast suspensions were subsequently isolated for testing. The fibroblasts were then exposed to sterile water solutions containing 0.25, 1.0, 2.5, or 4.0 μM MeHg. Gribble et al. (2005) found that MeHg caused a non-permanent delay in the G_2 phase stage in p53+ cells at MeHg concentration of 1.0 μM and above. There was also an inhibition of G_0/G_1 cells to progress to G_2. At 24 h following treatment, an average of 28 % of untreated control cells had progressed to the G_2 stage, compared with no progression to G_2 in 1.0 and 2.5 μm MeHg treated cells. In addition, it was found that1.0 μM MeHg caused an approximate 3-fold larger percentage of p53+ cells to undergo apoptosis than p53− cells at 24 and 48 h.

In a population of cells allowed to progress to the G_2 stage, treatment with a MeHg concentration of 2.5 μM resulted in a time-dependent inability of G_2 cells to progress in the cell cycle (Gribble et al. 2005). At 24 h, there was a 17.3 % increase in the number of p53+ cells over the untreated cells; and at 48 h, 77.0 % of the treated cells remained at the G_2 stage, compared with an average of only 7.3 % of the untreated G_2 cells.

Gribble et al. (2005) concluded that low-dose MeHg causes cell cycle inhibition in the absence of cytotoxicity. Their study results suggest a mechanism involving p53 signaling that may have important implications for neurodevelopmental toxicity associated with MeHg.

Burke et al. (2006) further investigated the phenomenon of cell cycle interference by conducting both in vivo and in vitro studies using Sprague-Dawley rats. In the in vivo study, rats were mated, and the litter birth date was considered to be post-natal day 0 (P0). At day P7, rats were injected subcutaneously with vehicle (controls) or 0.1–30 μg/g MeHg. Rats were sacrificed at 7 h, 24 h, or 2 weeks, and the hippocampus and cerebellum were removed from whole brains. Tissues for cell cultures were obtained at embryonic day 14.5 for cortical precursors or day P7 pups for granule cells. Acute changes in DNA synthesis were evaluated at 7 h post-dosing, using [^{3}H]-thymidine as a marker of DNA synthesis.

Burke et al. (2006) found that the incorporation of [^{3}H]-thymidine decreased as the concentration of MeHg increased above 0.1 μg/g in the hippocampus. However, DNA synthesis in the cerebellum was unchanged across the entire dosage range of MeHg. The authors suggested that this indicated a region-specific response to MeHg, as total Hg levels were very similar in both areas of the brain. They also pointed out that since brain development relies on the production of new neurons, the acute decrease in the synthesis of DNA in the hippocampus might have permanent effects. This is supported by the observation that MeHg exposure on P7 resulted in a decrease in the cell number at P21 by 17 %. In contrast, parallel studies of the cerebellum showed no significant change in cell number at 2 weeks post-exposure.

To determine whether the difference in the effects in the two brain regions was the result of differences in cellular characteristics, as opposed to regional differences, Burke et al. (2006) then conducted in vitro experiments. In isolated granule cells from the line that had shown resistance to MeHg in the in vivo studies, [^{3}H]

incorporation was decreased as the duration of exposure increased. Whereas 0.3 μM MeHg caused no change at 6 h, [^{3}H]-thymidine incorporation was decreased by 17 % at 24 h. Exposure of the granule cells to 3 μM MeHg resulted in a 43 % decrease at 6 h and 92 % at 24 h, indicating that granule cells are, in fact, responsive to MeHg, and they are not simply resistant to MeHg exposure.

Using bromodeoxyuridine (BrdU) labeling to assess the number of cells in S-phase during the last 2 h of MeHg incubation, Burke et al. (2006) found that in 6 h cerebellar granule cultures, MeHg induced a 73 % reduction in [^{3}H]-thymidine incorporation into DNA and a 43 % decrease in BrdU labeling. The authors noted that this is consistent with the hypothesis that MeHg inhibits the transition from the G_1 to the S phase of cell division. Based on the decrease in BrdU labeling, they also assessed the effects of MeHg on proteins involved in the transition of the G_1 to S-phase of the cell cycle. One of these regulatory proteins is cyclin E. Burke et al. (2006) found that cyclin E is specifically targeted by MeHg; thus the effects of MeHg they observed on DNA synthesis may be the result of the MeHg-induced reduction in the availability of cyclin E.

3.7 Effects on Glutathione (GSH) Activity

The brain is particularly sensitive to oxidative injury, in large part due to its high rate of oxidative metabolism (Halliwell 1992). In the cellular cytosol, GSH serves as the major antioxidant, helping to scavenge ROS.

In a study described earlier in this paper, Ndountse and Chan (2008) used GSH levels as an indicator of cytotoxicity, measuring GSH levels in cultured human neuroblastoma cells incubated at MeHg concentrations of 0.1–5 μM. They found that GSH was decreased, even at non-toxic concentrations of MeHg, noting that GSH depletion is an indicator of oxidative stress. They further noted that they had found a good correlation between the observed decrease in GSH and cell death.

In a review of the neurodegenerative effects of mercury and its compounds, Carocci et al. (2014) note that the most important mechanism by which mercury causes toxicity is by mitochondrial damage via depletion of GSH, coupled with thiol binding which in turn generates free radicals. Mori et al. (2007) point out that the function of mitochondrial GSH would be expected to be essentially the same as cytosolic GSH with respect to MeHg toxicity. In this study, both whole tissue GSH and mitochondrial GSH were lower in the rat brains than in the livers, and the same was true of two other antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX). This occurred despite the higher oxygen consumption and subsequent ROS production in the brain. The authors suggested that the brain mitochondria might therefore function with marginal stability against oxidative stress. Further, the mitochondria of the cerebellum might be particularly sensitive to this, because of their higher oxygen consumption and ROS production than the cerebrum. In fact, the GSH levels measured in mitochondria in the cerebellum of intact rats was as low as one-third that of cerebral mitochondria. Mori et al. (2007) noted that the higher susceptibility to MeHg-induced oxidative

damage in brain mitochondria (particularly in the cerebellum) compared with the liver mitochondria might account, at least in part, for the selective toxicity of MeHg. Mori et al. (2007) concluded that their data suggest that the high MeHg-induced activity in mitochondrial ROS generation and low activity in the brain's defense system, particularly in the cerebellum, would easily cause a critical imbalance in ROS production and at least partially account for the selective neurotoxicity of MeHg.

Franco et al. (2006) examined the exclusive contribution of MeHg exposure through maternal milk on biochemical parameters related to the thiol status in the cerebellums of suckling mice. The thiol status was determined by GSH levels and GPX and glutathione reductase (GR) activity. In this study, 14 Swiss albino mice dams were randomly assigned to one of two groups of seven females each (one treatment and one control group). Pups (eight per litter) were maintained with their mothers, half of which were immediately exposed to MeHg (10 mg/L) in drinking water (treatment group) or to MeHg-free tap water ad libitum (control group). The exclusive route of MeHg exposure for the treated offspring was maternal milk.

Franco et al. (2006) found that the GSH level in the cerebellum was significantly higher ($p < 0.05$) in the MeHg-exposed dams than in the control rats. In the exposed pups, however, the response was completely the opposite, with MeHg exposure through breast milk resulting in a significant decrease in GSH levels ($p < 0.05$). Cerebellar GR activity was also significantly higher ($p < 0.05$) in MeHg exposed dams than in controls, but this increase was not seen in exposed pups. A two-tailed Pearson correlation test revealed a significant positive correlation between cerebellar GR activity and cerebellar GSH in mothers ($p < 0.001$). MeHg exposure did not affect GPX. Franco et al. (2006) concluded that decreased motor performance in the MeHg-exposed pups may be related to alterations in the cerebellar thiol status. They suggest that the increases in GSH levels and GR activity in the cerebellums of MeHg-exposed dams could represent a compensatory response to the oxidative effects of MeHg toward endogenous GSH. Franco et al. (2006) also suggest that the inability of the pups to perform this compensatory response is probably due to the immaturity of the CNS in the pups, making them more susceptible to the oxidative effects of MeHg on cerebellar thiol status.

The effects of MeHg on microglia were examined using the murine N9 microglial cell line (Garg and Chang 2006). Microglia are macrophage-like cells that make up ~15 % of the cell population within the CNS. As such, they are responsible for removal of invading pathogens and are critical to the survival and maintenance of CNS neurons. They also secrete a number of proteins, such as IL-6, which can be either beneficial or detrimental to neuronal cells, depending on the internal conditions. To test for cytotoxicity, the cells were treated with various concentrations of MeHg for one day, and the viability of each treatment subsequently determined. Cytotoxicity was found to appear in a rapid and irreversible manner. Under the conditions of this experiment, MeHg at concentrations of 4, 8, 12, or 16 μM reduced cell viability by 12 %, 59 %, 85 %, and 95 %, respectively.

Targets of developmental MeHg exposure include neural cell adhesion molecules (NCAMs), which are sialoglyco conjugates whose proper temporal and spatial expression is important at all stages of neurodevelopment, especially during the formation of synapses. Dey et al. (1999) dosed rat pups subcutaneously with 7.0 mg/kg on every other day from postnatal days 3–13, and investigated the effects of MeHg on the temporal expression of NCAM during development. Postmortem examination of whole-cerebellum homogenates, cerebellar synaptosomes, and isolated cerebellar growth cones collected at postpartum days 15, 30, and 60 was conducted. Golgi sialytransferase activity analysis revealed significant reductions in samples collected at postnatal day 15; however, no such changes were found at postnatal days 30 or 60. In vitro studies revealed decreasing MeHg sensitivity of cerebellar sialytransferases with increasing developmental age. The authors concluded that MeHg-induced perturbation of the developmentally regulated expression of polysialylated NCAM during brain formation may disturb the stereotypic formation of neuronal contacts and contribute to the behavioral and morphologic disturbances seen following MeHg poisoning (Dey et al. 1999).

In a study of the in vivo degenerative effects of methylmercuric chloride on rat brain and cranial nerves, Kinoshita et al. (1999) demonstrated a disturbance in the integrity of microtubules and neurofilaments in the rat nervous system, particularly in the optic nerves. Specifically, electron microscopic examination revealed a marked decrease in microtubules and a moderate decrease of neurofilaments in the myelinated fibers of optic nerves in treated animals.

Faro et al. (2005) investigated the effect of pretreatment with GSH, cysteine, or methionine on MeHg-induced dopamine release from the rat striatum (Faro et al. 2005). Adult female Sprague-Dawley rats were administered a perfusion fluid containing either MeHg, MeHg following GSH pretreatment, MeHg following cysteine pretreatment, or MeHg following methionine pretreatment via a dialysis probe inserted directly into the striatum. The individual concentration of each of the four substances in the perfusion fluid was 400 μM. Infusion of 400 μM MeHg alone resulted in a 1941 % (±199 %) increase in extracellular dopamine levels (vs. basal levels). Infusion of 400 μM following GSH pretreatment resulted in an increase in extracellular dopamine of only 465 % (±104 %), or 76 % less increase than that caused by MeHg alone. MeHg infusion following pretreatment with cysteine resulted in only an increase of 740 % (±149 %), or only 62 % lower than that induced by MeHg alone. Treatment with MeHg following methionine pretreatment resulted in no significant difference from MeHg alone.

In a study previously described in the current paper, Faro et al. (2005) pointed out that MeHg promotes a decrease in intracellular GSH levels, as previously demonstrated by Choi et al. (1996). Thus, pretreatment with GSH would to some extent ameliorate the impact of MeHg on existing cellular GSH, as found in this study. They also noted that the amino acid-cysteine has a free -SH group, whereas the amino acid methionine has a sulfur atom without the capacity to form -SH groups. Therefore, it is not surprising that the -SH group available in the cysteine pretreated group would bind with MeHg and serve to decrease dopamine release. But since the

sulfur atom in methionine was unavailable for binding to MeHg, pretreatment with that amino acid had no effect on MeHg-induced dopamine release (Faro et al. 2005).

3.8 Involvement with Nitric Oxide Synthetase

Shinyashiki et al. (1998) examined the effects of MeHg on cerebral and cerebellar neuronal nitric oxide synthase (nNOS) isoforms in rat brain in vivo and in vitro. Eight week-old male Wistar rats were given subcutaneous doses of 10 mg MeHg/kg/day for 8 days. In vivo manifestation of neurotoxicity was considered to be hind limb crossing, which was evaluated as an indication of paralysis. Five animals were sacrificed each at 1, 2, 5, and 8 days after the first injection and when paralysis was achieved, and the cerebrum and cerebellum of each was removed and examined for MeHg levels. Effects on enzyme activity, interaction of NOS with mercuric compounds via thiols, and involvement of SH groups in MeHg-induced enzyme loss were examined in vitro.

Total Hg concentrations in blood, cerebrum, and cerebellum increased progressively through 8 days of exposure (Shinyashiki et al. 1998). The first hind limb crossing was seen 14.5 days after the first injection. The activity of nNOS increased in both the cerebrum and cerebellum, but in a different manner. In the cerebrum, nNOS activity increased significantly with time and peaked 5 days following the first injection ($p < 0.01$); it then declined slightly, but statistically significantly, at 8 days ($p < 0.01$). NOS activity in the cerebellum, however, was significantly increased only after 8 days ($p < 0.05$). In contrast to the in vivo results, in vitro tests showed a decrease in cerebellar nNOS activity in a concentration-dependent fashion following MeHg-induced covalent modification of thiol groups. Shinyashiki et al. (1998) suggested MeHg causes not only an increase in intracellular calcium, but also in nNOS protein, bringing about overproduction of NO.

Chuu et al. (2001) administered male mice oral gavage MeHg doses of 0.2 or 2.0 mg/kg/day for seven consecutive days. The animals were sacrificed by pentobarbital injection at 0, 5, and 11 weeks following cessation of treatment. Brainstem tissue was assayed for Na^+/K^+-ATPase activity immediately following the cessation of treatment and from animals sacrificed at 5 and 11 weeks post-treatment. Brainstem tissue and whole blood were also assayed using a nitric oxide (NO)/ozone chemiluminescence assay method. Their tests revealed MeHg-related inhibition of Na^+/K^+-ATPase activity in the brainstems of mice treated with 0.2 and 2.0 mg/kg MeHg, but this was observable only in those animals sacrificed immediately following treatment cessation; no such effect was seen in animals sacrificed at 5 or 11 weeks post-treatment. A significant ($p < 0.05$) and irreversible increase in brainstem NO level was seen at all times during the experimental course (0, 5, or 11 weeks post-treatment) at 2.0 mg/kg/day, compared to vehicle controls. No discernible change in nitric oxide level was seen at

any post-treatment analysis period in animals receiving the 0.2 mg/kg dose. The authors concluded that high-dose MeHg intoxication is associated with a decrease in functional Na^+/K^+-ATPase activity in the brainstem of affected animals, secondary to excessive production of NO, leading to oto-toxicity and hearing loss (Chuu et al. 2001).

3.9 *Effects on Glial Cells and Glutamate-Glutamine Uptake and Homeostasis*

Of the non-neuronal support cells in the CNS, astrocytes are the most abundant. They participate in neurotransmitter synthesis, help to maintain K^+ balance, assist in neuronal migration during development, play a prominent role in glutamate-glutamine homeostasis, and are a structural component of the blood-brain barrier.

To identify the mechanisms involved in astrocyte damage due to MeHg exposure, Yin et al. (2007) examined the effects of MeHg on glutamine uptake and expression of glutamine transporters in primary astrocyte cultures. Pre-treatment with 1 μM, 5 μM, or 10 μM MeHg for 30 min resulted in significant ($p < 0.001$) inhibition of glutamine uptake at both 1 and 5 min measurements at all three concentrations, compared to controls. The degree of inhibition was concentration, but not time, dependent. Differences in glutamate uptake with 10 μM MeHg was also significantly higher ($p < 0.001$) than uptake with 1 μM ($p < 0.001$) and 5 μM ($p < 0.05$) at both 1 and 5 min.

Yin et al. (2007) also investigated the molecular mechanisms associated with the effects of MeHg on glutamine uptake in cultured astrocytes by measuring the astrocytic amino acid transporter mRNAs by RT-PCR. Glutamine uptake is dependent upon several sodium-dependent transporters, including SNAT1, SNAT3, and ASCT2. MeHg treatment at 10 μM MeHg (but not at 1 μM or 5 μM) significantly ($p < 0.05$) reduced the mRNA expression of SNAT3 and ASCT2, but not SNAT1, when compared to controls. Yin et al. (2007) concluded that their data, when taken collectively, demonstrate an association between MeHg exposure and increased mitochondrial membrane permeability, alterations in glutamine/glutamate cycling, and oxidative injury resulting from increased ROS formation. They further suggested that the ultimate effect of MeHg is the initiation of multiple additive or synergistic mechanisms of a disruptive nature that lead to cellular dysfunction and cell death (Yin et al. 2007).

Farina et al. (2003a) examined the effects of MeHg on glutamate uptake by brain cortical slices. Two-month-old male Swiss Albino mice were given MeHg chloride equivalent to doses of 0, 10, or 40 mg/kg MeHg chloride in drinking water for 15 days and then sacrificed and the cerebral cortex removed for experimentation. The 40 mg/kg equivalent dosage, but not the 10 mg/kg dose, significantly ($p = 0.013$) decreased glutamate uptake in the cortical slices.

To evaluate the effects of in vivo MeHg exposure on glutamate release from synaptosomes and glutamate uptake by brain cortical slices, Farina et al. (2003b) divided 48 suckling Wistar rat pups into two groups of equal size. The pups received daily subcutaneous (s.c.) injections of MeHg dissolved in a 25 μM $NaHCO_3$ solution beginning on post-natal day 3 (PND 3). The 24 MeHg-treated rat pups were divided in three groups of eight pups each that were sacrificed on PNDs 10, 17, and 24 for preparation of synaptosomal and cortical preparations. The 2 mg/kg dosage was based on a prior study by Miyamoto et al. (2001), in which the vulnerability of developing cortical neurons to MeHg was examined. Control rats ($n = 24$) received daily s.c. injections of a 25 μM $NaHCO_3$ solution.

In another paper, Farina et al. (2003b) found that glutamate release increased with age in both control and treated animals. The control pups sacrificed on PND 24 had significantly ($p < 0.05$) elevated glutamate release levels as compared to the 10- and 17-day sacrificed animals. The 24-day-old MeHg-treated pups had glutamate release levels significantly higher than the 24-day controls ($p < 0.05$) and the 10- and 17-PND-sacrificed MeHg-treated pups ($p < 0.05$). In contrast with the increase in glutamate with age and MeHg treatment, glutamate uptake by cortical neurons was found to decrease with age in both controls and MeHg-treated animals. In cortical slices from pups sacrificed on PND 24, there was a significant ($p < 0.05$) increase (56 %) compared with age-matched controls. These authors concluded that their data suggest that the effect of MeHg on glutamate release from presynaptic nerve terminals could be involved in its neurotoxicity in suckling rats, and that the observed increase in glutamate uptake could correspond to a pathologic response to MeHg. Farina et al. (2003b) went on to suggest that it is possible that the neurologic deficits seen in MeHg-exposed children might be related, at least in part, to a MeHg-induced disturbance of glutamate homeostasis.

Due to the effects of MeHg on the brains of infants whose mothers had been exposed to MeHg during pregnancy, Manfroi et al. (2004) examined the effects of MeHg in weanling mice that had been exposed to MeHg only from the breast milk of their mothers. To do so, glutamate uptake by cerebellar slices from weanling mice was measured and compared with uptake from cerebellar slices from their mothers. While no differences found in glutamate uptake between exposed and control mothers, a significant ($p < 0.05$) decrease in glutamate uptake was observed in exposed weanlings, when compared to controls.

3.10 Other Mechanisms

Calpain is a Ca^{++}-dependent cysteine peptidase (protease) that was first identified in rat brain by Guroff (1963). It has been reported that deregulation of calpain activity can result in tissue damage and is associated with events such as stroke and brain trauma (Goll et al. 2003). Calpain degrades many specific substrates, including microtubule-associated protein 2, α-fodrin, and p35.

Lee et al. (2000) reported that the calpain/cyclin-dependent kinase 5 cdk5/p35 cascade is important in neuronal cell apoptosis. The cdk5 activator, specifically expressed in neuronal cells, causes a cleavage of p35 to p25, which is followed by the redistribution and overactivation of cdk5 in the presence of p25. Caplain-induced conversion of p35 to p25 leads to the formation of a cdk5-p25 complex, which results in the hyperphosphorylation and degradation of many proteins, including tau and neurofilaments (Nath et al. 2000).

Sakaue et al. (2005) explored a possible mechanism by which MeHg leads to cell death in rat cerebellar granule cells. Their hypothesis was that MeHg activates the calpain-dependent cascade and leads to granular cell death in the cerebellum. To investigate this, they looked specifically at the calpain/cyclin dependent kinase (cdk5/p35) cascade which, when activated, leads to neuronal cell apoptosis. In their studies, cultured cerebellar granule neurons prepared from Wistar rats were treated with MeHg at concentrations ranging from 0 to 1 μM (1000 nM) for 24 or 48 h with or without calpain inhibitor II. Intracellular Ca^{++} concentrations were determined using the fluorescent Ca^{++} indicator, Fluo-3 (Fluo-3 acetoxymethyl ester), which specifically binds to calcium ions.

Sakaue and colleagues found that MeHg induced death in cerebellar granule cells in a dose dependent manner. In investigating the first stage of the calpain-activated cell death, which is an upsurge in intracellular calcium after MeHg treatment, the intracellular calcium increased in a dose-dependent manner. Even at the low dosage of 30 nM MeHg, significant ($p < 0.05$) cell death was observed. However, treatment with calpain inhibitor II restored the viability of cells treated with 30 nM or 100 nM MeHg from 76 % to 89 % and from 43 % to 60 %, respectively. Thus, the inhibitory effect of calpain inhibitor II was demonstrated to be statistically significant ($p < 0.05$), but incomplete.

Sakaue et al. (2005) also investigated the effect of MeHg on rat neuroblastoma cells for 48 h, and found that the sensitivity of the neuroblastoma cells to MeHg was less than the sensitivity of cerebellar granule cells. The viability of the B35 cells was found to be significantly ($p < 0.05$) decreased only at MeHg concentrations above 300 nM.

Sakaue et al (2005) also measured the cleavage of α-fodrin, a known calpain substrate, and found that it occurred in a dose-dependent manner with the addition of MeHg. They further noted that calpain inhibitor II did not completely stop the cascade that lead to calpain activated apoptosis, although it did reduce it. Those authors concluded that the calpain cascade may be one of the mechanisms that contribute to microtubule degradation that leads to cell death.

Although tau proteins are necessary for microtubule stabilization, Sakaue et al. reported that it was improbable that the phosphorylation of tau contributed to MeHg-induced cell death at very low concentrations. Rather, proteins containing cytoskeletons seemed to degrade and cause dysfunction via calpain-digestion in the neuronal cells treated with a very low dose of MeHg (Sakaue et al. 2005).

4 Ethylmercury: Mechanisms of Toxic Action

Ethylmercury is the other alkyl mercury compound discussed in this paper. While there has been considerable interest in ethylmercury in recent years due to its presence in the body as the result of the in vivo metabolism of the preservative thimerosal, almost all of the data regarding its metabolism have come from the studies of thimerosal and not ethylmercury itself.

Sodium ethylmercuric thiosalicylate (thimerosal; Merthiolate) is a mercury-containing preservative used in some multi-dose vials of vaccines and other products since the 1930s. It has the chemical name ethyl(2-mercaptobenzoato-(2-)-O,S) mercurate(1-) sodium and contains approximately 49 % mercury by volume. Thimerosal is broken down by in vivo metabolic processes to ethylmercury (EtHg) and salicylic acid. Thimerosal is quickly metabolized in vivo, due to its reactions with protein and non-protein thiols (Wu et al. 2008).

4.1 Mobilization of Intracellular Ca^{++}

Thimerosal has been shown to be a versatile sulfhydryl reagent, a mobilizer of intracellular calcium, and a modulator of cell function (Elferink 1999). It has been shown to induce the release of intracellular calcium stores in many cell types. This mobilization of calcium can in turn modulate a number of cell functions. Tornquist et al. (1999) found that thimerosal mobilized sequestered calcium and evoked modest store-dependent calcium entry in thyroid FRTL-5 cells. The mechanism of action was suggested to be mediated via activation of protein kinase C, as thimerosal potently stimulated the bonding of [^{3}H]phorbol 12,13-dibutyrate and was without effect on store-operated calcium entry in cells treated with staurosporine or in cells with down-regulated protein kinase C. Whole-cell patch clamping experiments revealed that thimerosal did not depolarize the membrane potential. It was concluded that thimerosal attenuates any increase in internal calcium ion concentration, probably by activating a plasma membrane Ca^{++}-ATPase (Tornquist et al. 1999).

Thimerosal has also been shown to be a potent activator of intracellular calcium release in pig oocytes. Such activation mimics the effects of sperm-induced release of intracellular calcium, as well as other activation events that occur in pig oocytes (Machaty et al. 1999). Wang et al. (1999) examined the temporal relationship between intracellular calcium transients, cortical granule exocytosis, and the zona reaction induced by thimerosal. These researchers found that thimerosal induced the same degree of exocytosis in oocytes that was caused by sperm penetration. Further, the zona block to sperm penetration in thimerosal-treated oocytes occurred within 35 min of cortical granule exocytosis and within 40 min of the first calcium transient. Machaty et al. (1999) found that the thimerosal-induced Ca^{++} release did

not require the formation of IP_3. In addition, thimerosal destroyed the meiotic spindle, preventing further development.

Calcium channels in skeletal muscle, cardiac muscle, and certain nerve fibers have a high affinity for the plant alkaloid ryanodine (Sitsapesan and Williams 2000). The receptors for which ryanodine has this particular affinity are known as ryanodine receptors (RyR). Eager and Dulhunty (1999) found that thimerosal reacts with specific cysteine residues on RyR, contributing to either activation or inhibition of the channel, depending on the domain and particular class of cysteine associated with that receptor.

Using whole-cell patch clamping to study the effects of thimerosal on tetrodotoxin (TTX) sensitive and TTX-resistant sodium channels in dorsal root ganglion neurons, Song et al. (2000) found that thimerosal blocked the two channel types in a dose-dependent fashion. The inhibition was considerably more pronounced in the TTX-resistant channels, but the effect was not reversed in either case with washing with thimerosal-free solution. The thimerosal-induced inactivation of both types of sodium channels would serve to diminish neuronal activity.

As previously noted in this paper, inositol 1,4,5-triphosphate (IP_3) is involved in intracellular calcium homeostasis; and the binding of this compound to the inositol triphosphate receptor (IPR) is modulated by a number of compounds, including MeHg. IP_3 plays its vital role in calcium homeostasis through its action as a second messenger responsible for the release of Ca^{++} from intracellular stores following receptor activation. Further, binding to the IPR is modulated by a number of compounds, including MeHg. In an earlier study, Sayers et al. (1993) investigated the mechanism by which thimerosal affects the biochemical properties of intracellular Ca^{++} pumps and channels using rat cerebellar microsomes and rabbit skeletal muscle and fluorescence monitoring techniques. The effect of up to 50 μM thimerosal on IP_3 binding was assessed by the addition of thimerosal to cerebellar microsomes incubated with 40 nM [^{3}H]IP_3. In the rat cerebellar microsomes, a concentration-dependent inhibition of Ca^{++} uptake was observed, but 75 μM thimerosal was required for complete inhibition. In the rabbit skeletal muscle, the rate of Ca^{++} uptake by the sarcoplasmic reticulum was progressively decreased with increasing concentrations of thimerosal, with nearly complete inhibition occurring at thimerosal concentrations over 2 μM. Upon the addition of the Ca^{++} ionophore A23187 to enhance the permeability of the cell membrane to Ca^{++}, Ca^{++}-ATPase activity was completely inhibited by thimerosal, indicating that thimerosal had a direct, rather than non-specific, effect on the Ca^{++}-ATPase. Thimerosal concentrations up to 50 μM had no effect on IP_3 binding or on IP_3 metabolism in the cerebellar microsomal preparation. Sayers et al. (1993) concluded that the opening of the IP_3-sensitive Ca^{++} channel is a complex event, and that new models to explain IP_3-induced Ca^{++} release were necessary.

Since the report of Sayers et al. (1993), other researchers have investigated the effects of thimerosal on IP_3-mediated Ca^{++} release. Vanlingen et al. (1999) reported that the binding of IP_3 to its membrane receptors can be differentially modulated by thimerosal. Using a preparation of cerebellar microsomes, these researchers found that thimerosal had a stimulatory effect on the binding of IP_3 to IP_3R1 receptors.

Green et al. (1999) reported that the sensitivity of intracellular calcium stores to IP_3 increases the affinity of the IP_3 receptor in rat hepatocytes; and thimerosal was further shown to enhance agonist-specific differences in the oscillation of intracellular calcium in rat hepatocytes. Mason and Mahaut-Smith (2001) also reported the voltage-dependent Ca^{++} release in rat megakaryocytes following sensitization of IP_3 receptors with thimerosal.

Bultynck et al. (2004) compared the functional and molecular effects of thimerosal on IP_3R1 and IP_3R3 receptors. Using a culture of A7r5 embryonic rat aorta cells, which express primarily the IP_3R1 isoform, they found that thimerosal produced a modulated biphasic effect on the IP_3induced Ca^{++} release and IP_3-binding activity of IP_3R1 receptors. Thimerosal (1 μM) was found to strongly potentiate the IP_3-induced Ca^{++} release, and this was additive to the potentiation by the Ca^{++} itself. The authors stated that the additive effect of thimerosal and Ca^{++} may indicate that both agents cooperate to induce a conformational change to the IP_3R1 receptor that is much more sensitive to activation by IP_3. Bultynck et al. (2004) also studied the effects of thimerosal on R23-11 cells in culture. R23-11 cells are IP3R knockout cells derived from DT40 chicken B lymphoma cells by homologous recombination. These latter experiments showed that thimerosal potentiated IP_3-induced Ca^{++} release and the IP_3 binding activity of IP_3R1 in triple IP_3R knockout R23-11 cells, but not in the IP_3R3 isoform, which lacks the N-terminal suppressor domain. Bultynck et al. (2004) concluded that, collectively, their data revealed a thimerosal-dependent intramolecular interaction within the N-terminal domain of IP_3R1, and that this may be at least a part of the conformation changes occurring during the desensitization of IP_3R1 by thimerosal. Further, IP_3 may induce opening of the channel pore by modifying the interaction of the C-terminal channel domain with the N-terminal IP_3-binding domain.

4.2 Effects on Mitochondria (Membrane and ROS)

Much of the increase in intracellular Ca^{++} is attributable to its release from the mitochondria (Sharpe et al. 2012). Sharpe et al. (2012) investigated the effects of ethylmercury (from thimerosal) on the mitochondria of normal human astrocytes, paying particular attention to mitochondrial function and the generation of specific antioxidants. These investigators pointed out that the types and levels of antioxidant enzymes of human astrocytes are somewhat different from most other cell types.

In their experiments, Sharpe and his co-investigators incubated astrocytic cells for 1 h, before performing fixation of the cells. Thimerosal solutions were prepared to a maximum concentration of 360 μM, and 10 μL were added to a 240 μL astrocytic volume. Additional 10 μL aliquots were added at 10-min intervals. Measurements of DNA, oxidized DNA bases, and blunt-ended breaks were also performed. Concentration dependence was determined by adding Thimerosal concentrations of 0–14.4 μM to the cell medium at $t = 0$, 10, 20, 30, 40, and

50 min, before fixation at 60 min. ROS generation was measured by dichlorohydrofluorescein (DCF) fluorescence in isolated mitochondria.

Treatment with 14.4 μM thimerosal caused ~50 % decrease in mitochondrial membrane potential at 1 h, along with a 200 % increase in mitochondrially generated oxidants. Time course evaluation of ethylmercury-induced changes revealed that the generation of ROS is an early event in the course of events leading to apoptosis and occurred prior to changes in the mitochondrial membrane potential. Further, thimerosal-treated astrocytic mitochondria were observed to generate four times the amount of ROS as control mitochondria. In comparison, steady state generation of ROS in areas with no mitochondria was unchanged.

Sharpe et al. (2012) reported a 5-fold increase in levels of oxidant-damaged mitochondrial DNA and increases in levels of mito-DNA nicks and blunt-ended breaks. Mitochondrial DNA was found to be far more vulnerable to ethylmercury-induced damage, compared with nuclear DNA. These researchers observed a 240 % increase in the levels of mitochondrial DNA breaks, a 300 % increase in 3′OH DNA nicks, and a 460 % increase in the levels of oxidized bases (apurinic or apyrimidinic sites). Since mitochondrial DNA is localized within the matrix, it was concluded that this is the main site of ROS generation.

Sharpe et al. (2012) also noted that EtHg not only inhibits mitochondrial respiration, leading to a drop in the steady state membrane potential, but also causes concurrent increases in the formation of superoxide, hydrogen peroxide, and Fenton/Haver-Weiss generated hydroxyl radical.

4.3 Effects on Arachidonic Acid, Leukotriene Synthesis, and Membrane Integrity

The levels of free AA in cells are known to be maintained by a balance between hydrolysis from membrane phospholipids by phospholipase A_2 and reincorporation into phospholipids catalyzed by the L-type neutral amino acid carrier transport (LAT) system (Chilton et al. 1996). Thimerosal has been shown to be involved in the liberation and prolonged availability of arachidonic acid (AA) and AA metabolites in various mammalian tissues and cells (Chen et al. 2003; Hatzelmann et al. 1990; Kaever et al. 1988; Stuning et al. 1988; Zarini et al. 2006). Kaever et al. (1988) stated that the availability of arachidonic acid represents the fundamental prerequisite of cellular eicosanoid synthesis.

To examine the effects of thimerosal on μμLAT in human neutrophils, Zarini et al. (2006) isolated neutrophils from whole human blood, and incubated neutrophil microsomes and a mixture of standard lysophospholipids with AA Co-A in the presence or absence of 50 μM thimerosal. In the absence of thimerosal, the corresponding diacylphospholipids were formed. However, when the thimerosal was added to the incubated microsomes 2 min prior to the addition of the lysophospholipids, an almost complete inhibition of the formation of phospholipids

was observed. Subsequent experiments in which the thimerosal was removed prior to the addition of the lysophosphate mixture revealed that the thimerosal-induced inhibition was irreversible.

Zarini et al. (2006) concluded that irreversible covalent binding of thimerosal to LAT inhibits activity of the LAT enzyme and the reacylation of AA into membrane phospholipids.

Arachidonic acid (AA) is an important constituent of the phospholipid component of mammalian cell membranes, and is present in esterified form in phospholipids and triglycerides in the membranes (Stuning et al. 1988). In vivo, 5-lipoxygenase in the cytosol translocates to the nuclear membrane and associates with 5-lipoxygenase activating protein (FLAP), which is believed to act as an AA transfer protein that presents the substrate to the 5-lipoxygenase (Hardin and Limbird 2001).

In the lab, AA can also be released from membranes upon cell activation by the Ca^{++}-ionophore A23187. After release, AA may undergo oxidative metabolism through the enzyme cyclooxygenase, resulting in the formation of prostaglandins; through the cytochrome P450 system to produce a variety of metabolites including 19- or 20-hydroxy arachidonate and eicosatrienoic acids; or through lipoxygenases, including 5-lipoxygenase. The 5-lipoxygenase is one of the most important lipoxygenases, since it leads to the synthesis of leukotrienes (Hardin and Limbird, 2001). (See Fig. 1).

Because of its strong affinity of EtHg for SH groups, thimerosal is known to cause the inhibition of, among other enzymes, 5-lipoxygenase. In the presence of thimerosal, the metabolism of AA by human polymorphonuclear leucocytes resulted in the inhibition of 5-lipoxygenase, acetyltransferase, and the omega-oxidative system.

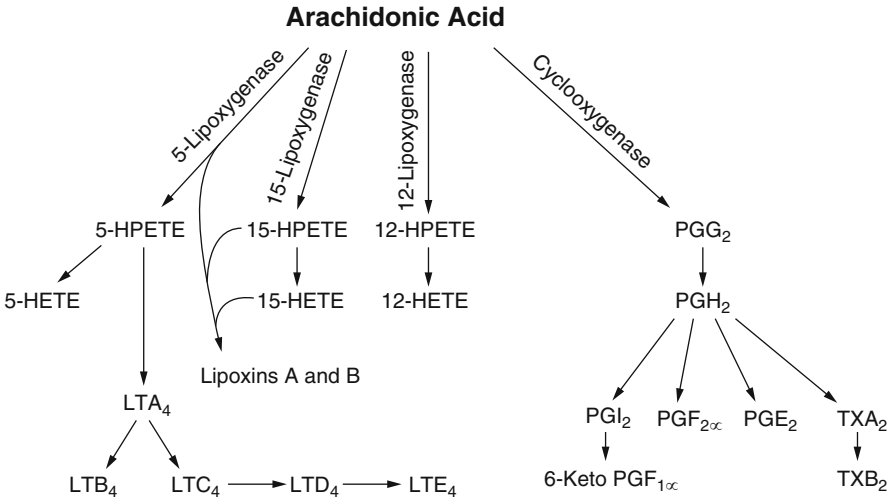

Fig. 1 Pathways of leukotriene and prostaglandin synthesis from arachidonic acid (AA). Source: Collectively from Samuelsson (1982, 1983), and Samuelsson et al. (1987)

Stuning et al. (1988) investigated the effects of thimerosal on the transformation of AA via the 5-lipoxygenase pathway in human leukocytes stimulated with A23187. In their experiments, they collected blood from healthy human donors to isolate polymorphonuclear leukocytes (PMNs), which they incubated with Ca^{++}-ionophore (usually 7.3 μM) in the presence of 2 mM Ca^{++}. Thimerosal dissolved in phosphate buffered saline (PBS) was then added at times ranging from 0 to 0.5 min before stimulation, then allowing the reaction to proceed for 10 min. at 37 °C. In other experiments, ^{14}C-labeled AA was given simultaneously with the stimulus. Separately, the metabolism of 3H-labeled LTB_4 was analyzed using HPLC.

Stuning et al. (1988) found that the polymorphonuclear leukocytes (PMN) stimulated with the Ca-ionophore A23187 released a variety of arachidonic acid metabolites in a concentration dependent fashion. These metabolites included LTB_4, LTC_4, and several analogs of LBT_4.

Thimerosal was found to exert a profound effect of the rate of formation of LTB_4. Pre-incubation of the cell suspension with thimerosal 0–5 min prior to the stimulation with the Ca^{++}-ionophore did not significantly alter the effect of thimerosal. Investigation of the effects of thimerosal on reacylation of AA into phospholipids and the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 5-hydroeicosatetraenoic acid (5-HETE) revealed that formation of labeled phospholipids, 5-HPETE, and 5-HETE was also decreased with increasing amounts of thimerosal. The concentration of the Ca^{++}-ionophore stimulus did not significantly influence the effects of thimerosal.

The inhibition of the omega-oxidation system by thimerosal was further confirmed in experiments in which PMNs were incubated with (^3H)LTB_4. Pre-incubation with thimerosal at a concentration >31 μM, previously demonstrated to cause a marked decrease in the formation of omega-oxidation products, resulted in a 4.3 (±0.3)-fold decrease in omega oxidation of (^3H)LTB_4 (Stuning et al. 1988). Stuning et al. (1988) further suggested that the interaction of environmental mercury with biological systems may have important implications regarding the efficacy of leukotriene synthesis.

4.4 Effects on Glutathione (GSH)

Muller et al. (2001) investigated the interaction of thimerosal with glutathione-S-transferases T1 (GST T1), an enzyme expressed solely in human erythrocytes and which displays genetic polymorphisms. Blood samples were collected from three (2 male, 1 female) healthy Caucasian adults previously identified as being either a non-conjugator of GST T1 (no enzyme activity), a normal conjugator (medium GST T1 activity), or super-conjugator (high GST T1 activity). Next, in vitro experiments were conducted using human erythrocytic and polymorphic GST T1 in the presence of a methyl chloride (MeCl) substrate and using an HPLC-fluorescence detection assay. In the case of the non-conjugator, thimerosal showed no effect due to the absence of GST T1. For the normal conjugator, a 2.77 mM

thimerosal concentration (1.12 mg/L) was found to inhibit 50 % of GST T1 activity; and 2.3 mM (0.93 mg/L) thimerosal resulted in a 50 % inhibition in the blood of the super-conjugator. A 14.8 mM (6 mg/L) thimerosal concentration led to residual enzyme activities equal to those of the non-conjugator.

When Muller et al. (2001) used a 0.6 mM concentration of thimerosal and 10,000 ppm MeCl concentration in the incubation medium, a 30 % inhibition of GST T1 was observed for both the normal and super-conjugator blood, compared with no effect for the non-conjugator blood. The authors concluded that the observed enzyme kinetics suggested a non-competitive inhibition of the human erythrocytic GST T1 enzyme and that sufficiently high concentrations of thimerosal may be able to change the phenotypic status of an individual by inhibition of the GST T1 enzyme, at least in vitro.

Wu et al. (2008) studied the importance of GSH in modulating the activity of thimerosal in experiments using human leukemia K562 cells and found that thimerosal undergoes an exchange reaction with cysteine, GSH, and human serum albumin and forms an EtHg adduct with single stranded DNA. In addition, the effect of thimerosal as a poisoning mechanism for topoisomerase H-alpha was also investigated as a mechanism of cytotoxicity in dividing cells. Topoisomerase is required to catalyze the double-stranded cleavage of DNA, allowing passage of a second DNA duplex through the break. The decatenation of DNA is known to be a topoisomerase H-specific reaction (Haldane and Sullivan 2001).

In their experiments, Wu et al. (2008) mixed 500 μL of 100 μM thimerosal with a similar quantity and concentration of cysteine in 50 % methanol (v/v) at room temperature for 5 min, followed by the addition of 10 μL of 6 % acetic acid (v/v). The same reaction of thimerosal with cysteine was carried out, with the exception of the addition of acetic acid. Wu et al. (2008) determined the ability of thimerosal to inhibit topoisomerase using a spectrofluorometric decantation assay as described in Hasinoff et al. (2006).

Wu et al. (2008) found that the reaction of thimerosal with cysteine produced a cysteine-EtHg adduct, and the reaction of thimerosal with GSH produced a GSH-thimerosal adduct, collectively confirming the ability of thimerosal to react with cysteine or GSH to produce a thiol-EtHg adduct and thiosalicylate. Given the high affinity of EtHg for thiols and the involvement of topoisomerase in the cleavage of double-stranded DNA, Wu et al. (2008) decided to investigate whether thimerosal could, in part, exert its cytotoxicity by inhibiting topoisomerase H-alpha through reacting with its free cysteine groups. In this part of their study, they found that thimerosal strongly inhibited the decatenation activity of purified topoisomerase H-alpha.

As the next step, Wu et al. (2008) decided to test whether GSH could protect isomerase H-alpha from the inhibitory effects of thimerosal. By adding various concentrations of GSH top topoisomerase H-alpha prior to thimerosal treatment, they found that an intracellular GSH level of 0.1–5 mM completely protected topoisomerase H-alpha decatenation activity from 5 to 20 μM thimerosal-induced inhibition.

In investigating the binding of thimerosal to DNA to induce DNA damage as a mechanism of cytotoxicity, Wu et al. (2008) found that treatment if K562 cells with thimerosal for 2 h resulted in a concentration-dependent reduction in remaining double-stranded DNA that achieved significance at all thimerosal concentrations of 50 of μM or higher, compared with cells not treated with thimerosal. From this, the authors concluded that thimerosal rapidly induces DNA damage in K562 cells. Wu et al. (2008) determined that these results suggest that the EtHg-thimerosal adduct did not significantly exchange with the free cysteines on topoisomerase H-alpha to inhibit it, and that the protective effect of GSH is consistent with thimerosal inhibiting topoisomerase H-alpha by binding to its free cysteines.

To assess the effects of lowering GSH levels on thimerosal-induced cytotoxicity, K562 cells in exponential growth were seeded (i.e., spread in a defined amount of cell suspension onto a plate) to yield a cell count of approximately 12,000 cells per well. At 24 or 48 h after seeding, cells were treated in the presence or absence of either 5 mM (−)-2-oxo-4-thiazolidinecarboxylic acid (OTC) or 100 μM buthionine sulfoximine (BSO) and allowed to grow another 24 or 48 h, before treatment with vehicle or various concentrations of thimerosal, and allowed to grow another 72 h. (OTC is a non-thiol precursor of cysteine that is converted intracellularly to cysteine, whereas BSO is known to inhibit the rate-limiting enzyme for GSH synthesis.). The levels of OCT and BSO used in this experiment did not cause measurable cytotoxicity. However, BSO or OTC pretreatments followed by exposure to various concentrations of thimerosal for 72 h were remarkable. Cell growth was inhibited by thimerosal with an IC_{50} value of 2.0 for untreated cells. For the OTC-treated cells, this value was increased 1.3-fold. For the BSO-treated cells, the LC_{50} value was reduced 16.7-fold. A *t*-test of three IC_{50} determinations showed that the small OTC-dependent increase in IC_{50} was not significant ($p = 0.22$), whereas the BSO-dependent decrease in IC50 was highly significant ($p < 0.0001$), leading Wu et al. (2008) to conclude that GSH partly protected the thimerosal-treated cells from growth inhibition.

Overall, Wu et al. (2008) concluded that their studies showed that thimerosal reacts rapidly with cysteine, GSH, HSA, and single-stranded DNA to form EtHg adducts detectable by mass spectrometry. Further, they showed thimerosal to be a potent inhibitor of the decantation activity of topoisomerase H-alpha, and that depletion of GSH with BSO greatly increased the growth-inhibitory effects of thimerosal.

4.5 Effects on Glutamate Transport

Mutkus et al. (2005) studied the effects of thimerosal on the transport of the non-metabolizable glutamate analog [3H]-D-aspartate in Chinese hamster ovary (CHO) cells. In these experiments, the CHO cells were transfected with two glutamate transporter subtypes: GLAST (EAAT1) and GLT-1 (EAAT2) (EAAT = excitatory amino acid transport system). Mutkus et al. (2005) also report

the results of studies to determine the effects of thimerosal on mRNA and protein levels of the glutamate transporters. The mutant CHO-K1 cell line DbB7 was chosen for their study since those cells possess essentially no endogenous Na^+-dependent glutamate transport.

When GLAST- and GLT-1 transfected CHO cells were treated with 0, 10, or 20 μM thimerosal, none of the treatment levels caused a discernable change in GLAST mRNA expression. Under the same conditions, however, GLT-1 mRNA expression was significantly ($p < 0.05$) in 10 μM treated cells (126 % of controls); but no such change was observed in 20 μM-treated cells.

In tests to examine glutamate transporter protein levels in thimerosal-treated CHO cells, Mutkus et al. (2005) found that 10 μM thimerosal caused a statistically significant ($p < 0.001$) increase in GLAST protein expression (198 % of controls); however, 20 μM thimerosal GLAST levels were not significantly different from controls. In contrast, GLT-1 protein levels were reported to decrease in a concentration-dependent manner in 10 and 20 μM thimerosal-treated CHO cells. However, the 10 μM cells were not distinguishable from controls, whereas the 20 μM-treated cells showed significant ($p < 0.001$) reductions at 20 μM treatment levels.

In another experiment, Mutkus et al. (2005) examined [^{3}H]aspartate uptake following 6 h of pretreatment with 0, 10, or 20 μM thimerosal, followed by measurement of [^{3}H]aspartate uptake during the subsequent 1, 5, 15, and 30 min. These experiments demonstrated that thimerosal caused a significant decrease in the uptake of [^{3}H]aspartate in transfected GLAST- and GLT-1 CHO cells. GLAST activity was significantly inhibited ($p < 0.01$) at the 1- and 5-min measurements with both 10 and 20 μM thimerosal. In the 10 μM treated cells, [^{3}H]aspartate uptake had recovered, but the decrease remained significant ($p < 0.01$) in 20 μM-treated cells. At the 30-min measurement, both 10 and 20 μM cells were back to control levels. The effects of thimerosal on [^{3}H]aspartate uptake in GLT-1 transfected cells was even more pronounced. The reduction in uptake was significant ($p < 0.01$) at all measurement times with both 10 and 20 μM treatment cells, with the sole exception of the 10 μM cells at the 30-min measurement.

The results of the Mutkus et al. (2005) experiments indicated that thimerosal treatment caused significant, selective changes in both glutamate transporter mRNA and protein expression in CHO cells. Thus, those authors concluded that their studies suggested that the application of thimerosal to the central nervous system might contribute to dysregulation of glutamate homeostasis.

4.6 Effects on Neurotransmitter Availability

Ida-Eto et al. (2011) administered thimerosal (1 mg/kg) intramuscularly to pregnant rats on gestational day 9, reported to be a susceptible time for the development of the fetal serotonergic system. Fetal serotonergic neurons were assessed at embryonic day 15, using anti-serotonin antibodies. A 1.9-fold increase in the number of serotonergic neurons in the lateral portion of the caudal raphe was observed in

thimerosal group compared to control values ($p < 0.01$). The authors concluded that their results indicated that embryonic exposure to thimerosal affects early development of serotonergic neurons.

To put the results of Ida-Eto et al. (2011) in proper perspective, the amount of mercury in a single syringe of vaccine from a multi-dose vial containing thimerosal as a preservative is ~0.25 μg/injection. If this is divided by the weight of a female human (assumed to weigh 60 kg), the dose to a pregnant woman would be ~0.43 μg/kg bw, as opposed to the 1 mg/kg injected into pregnant rats by Ida-Ito et al. Thus, the dosage administered to the rats was ~2500× as high as the human dosage. This study shows that sufficiently high doses of thimerosal/EtHg injected at precisely the critical time in development can enhance overall serotonin (5-hydroxytryptamine, or 5-HT) availability; however, the specific relationship to amounts of thimerosal administered at any time in pregnancy is unclear. In addition, the unrealistically high mercury doses themselves limit the relevance of the Ida-Eto findings to potential mechanism, and not necessarily to the toxicity of mercury from thimerosal in vaccines.

In a follow-up study, Ida-Eto et al. (2013) administered thimerosal to pregnant rats at dosages of 1.0, 0.1, or 0.01 mg Hg/kg by intramuscular injection on embryonic day 9. At the 1 mg Hg/kg dosage, most of the pups were dead soon after birth. No major anomalies, growth retardation, or reduced number of delivered were seen at the other two dosages. On day 50 of life, male offspring were killed and serotonin (5-HT), dopamine (DA), and their metabolites 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) concentrations in brain tissue were measured. Females were not used, since brain serotonin levels are influenced by the estrus cycle. Concentrations of hippocampal 5-HT and striatal DA in the male brains were measured by HPLC. Hippocampal 5-HT was significantly increased in both the 0.01 mg Hg/kg ($p < 0.05$) and 0.1 mg Hg/kg ($p < 0.01$) treatment groups, compared with controls. Striatal DA levels were also significantly increased in the 0.01 mg Hg/kg ($p < 0.05$) and 0.1 mg Hg/kg ($p < 0.01$).treatment groups vs. controls.

Ida-Eto et al. (2013) also found that the 5-HT metabolite 5-HIAA was increased in the 0.01 mg Hg/kg treated group, but the increase was not significant when compared with controls. The increase in the 0.1 mg Hg/kg vs. controls was significant ($p < 0.05$), however. The concentrations of striatal DOPAC and HVA were not significantly statistically different from controls. Those authors concluded that embryonic exposures to thimerosal at the concentrations tested produced lasting impairment of the brain monoaminergic system.

5 Summary and Conclusions

There are many commonalities/similarities in the mechanisms of toxic action of methylmercury and ethylmercury (from thimerosal). Thimerosal is quickly metabolized in vivo, due to its reactions with protein and non-protein thiols

(Wu et al. 2008), so the effects of thimerosal reported in numerous articles is very likely the result of exposure to the metabolite ethylmercury.

Evidence for the similarity of the various mechanisms of toxicity include the following:

- Both MeHg and EtHg bind to the amino acid cysteine (Clarkson 1995; Wu et al. 2008).
- Both MeHg and EtHg promote the release of arachidonic acid, resulting (on a potentially positive side) in a marked increase in leukotriene production. However, both MeHg and EtHg strongly inhibit the reacylation of arachidonic acid, thus inhibiting the reincorporation of this fatty acid into membrane phospholipids (Shanker et al. 2002; Verity et al. 1994; Zarini et al. 2006).
- Both decrease glutathione activity, thus providing less protection from the oxidative stress caused by MeHg and EtHg (Carocci et al. 2014; Ndountse and Chan (2008); Choi et al. 1996; Franco et al. 2006; Mori et al. 2007; Muller et al. 2001; Ndountse and Chan 2008; Wu et al. 2008).
- Both cause an increase in NOS, causing an overproduction of NO (Chen et al. 2003; Chuu et al. 2001; Shinyashiki et al. 1998).
- Both disrupt glutamate homeostasis (Farina et al. 2003a, b; Manfroi et al. 2004; Mutkus et al. 2005; Yin et al. 2007).
- Both cause oxidative stress/creation of ROS (Dreiem and Seegal 2007; Garg and Chang 2006; Myhre et al. 2003; Sharpe et al. 2012; Yin et al. 2007).
- Both alter intracellular calcium homeostasis (Elferink 1999; Hare et al. 1993; Kang et al. 2006; Limke et al. 2004b; Machaty et al. 1999; Marty and Atchison 1997; Minnema et al. 1987; Peng et al. 2002; Sayers et al. 1993; Sirois and Atchison, 2000; Szalai et al. 1999; Tornquist et al. 1999; Zarini et al. 2006).
- Both cause effects on cell division by damaging the spindle apparatus during mitosis (Burke et al. 2006; Castoldi et al. 2000; Gribble et al. 2005; Kim et al. 2007; Ou et al. 1999b; Machaty et al. 1999; Rodier et al. 1984).
- Both cause effects on receptor binding/neurotransmitter release involving one or more transmitters (Basu et al. 2008; Coccini et al. 2000; Cooper et al. 2003; Fonfria et al. 2001; Ida-Eto et al. 2011; Ndountse and Chan 2008; Yuan and Atchison 2003).
- Both cause DNA damage or impair DNA synthesis (Burke et al. 2006; Sharpe et al. 2012; Wu et al. 2008).

The difference in toxicity between MeHg and EtHg is likely the result of the more rapid metabolism and elimination of EtHg than MeHg and the amount of the mercurial form to which significant exposure is most likely to occur (i.e., MeHg exposure from frequent fish consumption vs. very small and widely spaced exposure to EtHg through thimerosal in multi-dose vials of vaccine).

Disclaimer The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Agency for Toxic Substances and Disease Registry.

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Arsenic Induction of Metallothionein and Metallothionein Induction Against Arsenic Cytotoxicity

Mohammad Tariqur Rahman and Marc De Ley

Contents

1 Introduction

Several factors contribute to human exposure to Arsenic (As), such as As-compounds in ground water, sodium arsenate (Na-As^{V}) in pesticides, and cigarette smoke. In natural waters, As is mostly found in inorganic forms as oxyanions of trivalent arsenite (As^{III}) or pentavalent arsenate (As^{V}) (Smedley and Kinniburgh 2002). Millions of people are exposed to As concentrations that are above the WHO recommended limit (Garelick et al. 2008; Hashim and Boffetta 2014). In the environment, oxidation states of As vary such as −3, 0, +3 and +5. Organic forms of As are mainly produced by biological activities.

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Depending upon the chemical form, the acute toxicity of arsenicals decreases from inorganic AsIII > inorganic AsV ≫ organic arsenicals (e.g., monomethyl AsV) (Klaassen 1990).

Various tissues and organs are affected by As toxicity, such as the skin, liver, kidneys and lungs. In those tissues/organs, a number of mechanisms have been identified resulting in cytotoxicity (Table 1). At the same time arsenicals were found to be associated with both in vitro and in vivo induced expression of several antioxidant defense systems including glutathione (GHS) and metallothionein (MT) (Table 2). It is important to note that MT is a family of proteins in which 12 members (isoforms) were reported to be actively expressed in mammals (Mehus et al. 2014).

The most important physiological importance of MT, the SH-containing family of low molecular weight proteins, lies in its toxic heavy metal detoxification and essential heavy metal homeostasis (reviewed by Hamer 1986). At the same time MT provides protection against reactive oxygen species (ROS) (Chiaverini and De Ley 2010; Kassim et al. 2013), which is one of the major primary toxic impacts of As exposure (Pi et al. 2002; Kitchin and Ahmad 2003; Valko et al. 2005). Elevated levels of ROS cause oxidative damage within a cell. Such oxidative stress is often directly associated with Zn deficiency (Kloubert and Rink 2015). Zn plays its antioxidant role as a cofactor of the superoxide dismutase (SOD) by modulating the glutathione (GSH) metabolism and MT expression, by competing with iron and copper in the cell membrane and also by inhibiting the nicotinamide adenine dinucleotide phosphate-oxidase enzyme (Cruz et al. 2015). Thus MTs, a major Zn homeostatic group of proteins, participate in controlling intracellular oxidative stress.

Once ingested through drinking water or food, As travels to organs such as the skin, kidneys and the liver through the circulatory system where MTs play an important role in response to toxic levels of As. However, little is known about the association of As toxicity and blood MT. It is expected that the plasma and cells of the circulatory systems including erythrocytes, thrombocytes, lymphocytes and their precursors are well known reservoirs as well as producers of MT (Vandeghinste et al. 2000; Rahman and De Ley 2001; Rahman and De Ley 2008; Maghdooni Bagheri et al. 2011).

The current review highlights the involvement of MT, with more focus on MT synthesis in blood, and in response to As. Finally, we propose how Zn inducible MT might provide protection against As toxicity in blood.

2 Metallothionein Induction and the Role of As

The increase of MT in plasma, bone marrow, erythrocytes, liver, kidneys differs markedly depending on the type and/or the dose of inducer(s). Dietary Zn for example, increases erythrocyte MT more than plasma MT (Grider et al. 1990), again endotoxins induce plasma MT more than the erythrocyte MT (Bremner

Table 1 Mechanism of cytotoxic effect of Arsenic

Effect in cellular physiology/ metabolism	Form of As	Effected/ treated cells/ tissues	(Possible) Impact	Reference
Disruption of oxidative phosphorylation by substituting phosphate in the formation of ATP	As^{V}	–	–	Bhuvaneswaran (1979)
Formation of metal-thiol complex in vicinal Cys of enzymes such as pyruvate dehydrogenase	As^{III}	–	–	Brown et al. (1976) Farrer et al. (2000)
Oxidative DNA damage (ODD)	As^{III}			Kessel et al. (2002)
Increased MMP-2 and MMP-9 activity, Cox-2 expression, and increased rate of cell proliferation of kidney stem cells	As^{III}	Kidney	Kidney cancer	Tokar et al. (2013)
Inhibition of blood δ-aminolevulinic acid dehydratase (ALAD) activity and glutathione (GSH) level	Na-As^{III}	Blood	–	Agrawal et al. (2014)
Increase of ROS and glutathione peroxidase (GPx) activity accompanied by a decreased SOD, CAT and reduced and oxidized glutathione (GSH and GSSG) levels in blood	Na-As^{III}	Blood	–	Agrawal et al. (2014)
Imbalance of pro-/anti-oxidants due to increased ROS thus disruption of the tyrosine kinase Src mediated transcription signalling pathway resulting in transcription of inflammatory cytokines.	As^{III}	Blood	Intensified inflammation	Milnerowicz et al. (2015)
Necrosis with partial apoptosis of macrophages causing release of TNF α at a cytotoxic dose.	As^{III} (5 μM) As^{V} (500 μM)	Macrophage	Carcinogenesis, hepatomegaly, and splenomegaly	Sakurai et al. (1998)

(continued)

Table 1 (continued)

Effect in cellular physiology/ metabolism	Form of As	Effected/ treated cells/ tissues	(Possible) Impact	Reference
ODD is significantly increased in double knock out [MT-1/MT-2] embryonic stem cells compared to the wild type cells	$NaAsO_2$	MT-$1^{-/-}$MT-$2^{-/-}$Embryonic SC	–	Qu and Waalkes (2014)
As induced toxicity in a dose-dependent fashion, by causing fragmentation of DNA, decreased mitochondrial membrane potential, increased intracellular GSH concentration	As^{III}	Rat kidney tubular cell	As-induced apoptosis has been attributed to the intracellular GSH reactive oxidation	Jimi et al. (2004)

Table 2 Expression of MT in response to As

Mode of As exposure	Effect on MT expression	Site of MT expression	Observation/conclusion	Reference
SC As^{III} injection in Rat	↑ MT accumulation ↑ MT-1, 2 mRNA	Liver	As inducible MT expression could be either directly by inducing transcription, or indirectly by post-transcriptional modifications	Albores et al. (1992)
SC injection in Mice As^{III}, As^{V}, MMA^{V}, DMA^{V}	↑ MT accumulation	Liver	Compared to As^{III}, 3-, 50-, and 120-fold higher molar amounts of As^{V}, MMA^{V}, and DMA^{V}, respectively are required for similar increase of hepatic MT content	Kreppel et al. (1993)
SC injection of in Mice As^{III}	↑ MT-1 mRNA	Kidney, spleen, stomach, intestine, heart, lung	MT transcription induction profile by As^{III} is similar to that of Zn or Cd	Kreppel et al. (1993)
As exposure from the environment	↑ MT-3 protein	Human epidermis of squamous cell carcinoma, basal cell carcinoma, and melanoma	High level of MT-3 protein in in cancerous human epidermis of arsenecosis patients correlates As exposure and the skin disorders and related cancers.	Slusser et al. (2014)

(continued)

Table 2 (continued)

Mode of As exposure	Effect on MT expression	Site of MT expression	Observation/conclusion	Reference
In vitro 6-7 μM of As^{III}	↑ MT-1X, 1 F, 2A, 3 mRNA ↓ MT-1A, 1E mRNA	Human astrocytoma (glioblastoma) cell line U87 MG	The increased MT1X, MT1F and MT2A transcription in human glioblastoma cells represent brain tumour acquired resistance to As cytotoxicity while the MT3 increase was suggested to be involved in arsenic-related induction of type II cell death	Falnoga et al. (2012)
In vitro $NaAsO_2$	↑ MT protein ↑ MT mRNA	Embryonic stem cell	As caused concentration-dependent increased expression of MT, and MTF-1	Qu and Waalkes (2014)
In vitro As^{III}	↓ MT protein	Mouse 3 T3 fibroblasts IκB kinaseβ $^{-/-}$	Activation of MKK4-c-Jun NH(2)-terminal kinase pathway, c-Jun phosphorylation, and apoptosis	Peng et al. (2007)
As^{III}	↑ MT-1 mRNA	Mouse *hepa1c1c7* cells	MT1 transcription is induced through MTF1. As ^{III} induction of MT-1 mRNA is lost in MTF1$^{-/-}$ cells. As ^{III} also induces MTF1 binding to the MRE of MT-1	He and Ma (2009)
As^{III} (50 μM) for 24 h	↑ MT protein	5 mM of α-lipoic acid (antioxidant) pretreated HepG2 cells, (for 8 h)	α-Lipoic acid pre-treatment increased MT expression and down-modulates Nrf2 mediated response	Huerta-Olvera et al. (2010)
In vitro As^{III} treatment	↑ MT protein	Rat lung fibroblasts	Dose-dependent disassembly of cellular microtubules, enhanced free tubulin pool and suppression of microtubule associated proteins (MAPs)	Zhao et al. (2012)
Inorganic As^{III} (500 nM)	↑ MT-1 and MT-2 ↑ SOD-1	Kidney stem cells	Transform rat kidney stem cells and partially differentiated progenitor cells to cancer cells	Tokar et al. (2013)

↑ = increase, ↓ = decrease, SC = subcutaneous, As^{III} = arsenite, As^{V} = arsenate, MMA^{V} = monomethylarsenate, and DMA^{V} = dimethylarsenate, MTF-1 = metal-responsive transcription factor-1 MRE = metal response element

et al. 1987). Huber and Cousins (1993) have shown that bone marrow MT expression is highly responsive to the amount of Zn in the diet.

As^{III} enters mammalian cells through multiple routes such as aquaglyceroporins (AQP), organic anion transporting polypeptides (OATP) as well as the glucose permeases namely, GluT1, GluT2 and GluT5 (Porquet and Filella 2007; Reviewed by Maciaszczyk-Dziubinska et al. 2012). Erythrocytes and lymphocytes, the two most abundant cell populations among all the cellular components of the circulatory system, express the highest level of GluT1 (Mueckler et al. 1985; Rathmell et al. 2000). Leukocytes as well as liver, spleen, and testis express AQP9, thus they mediate most As^{III} uptake from blood to liver (Ishibashi et al. 2009). In eukaryotic cells, As^{V} uptake is mediated by the high-affinity phosphate transporter, namely sodium-phosphate cotransporter (NaPiIIb), which is expressed in a variety of cells, such as the brush borders of enterocytes apical pole of alveolar type II cells in the lung, apical membrane of the mammary glands, epididymis cells of the testis, hepatocytes and apical cells of the renal proximal tubule (Murer et al. 2004).

Once As^{III} enters the cytoplasm, it is sequestrated by GSH and transported through ATP-binding cassette (ABC) transporters present at the plasma membranes. In mammals, inorganic As^{III} is methylated, the methylated forms are then exported from the cells by multiple ABC transporters, AQP and glucose permeases (Drobná et al. 2010; McDermott et al. 2010; Carew et al. 2011). In addition, multidrug resistance-associated protein (MRP), also called canalicular multispecific organic anion transporter (MRP1 and MRP2), transports GSH-As^{III} complex (Leslie et al. 2004). MRP2 mediates the efflux of seleno-bis(*S*-glutathionyl) arsinium ion. As^{V} in cytoplasm undergoes a rapid reduction to As^{III} and follows the similar fate of exportation through glucose permeases or MRP1 and 2 (Carew and Leslie 2010).

Major metal MT inducers such as Zn transportation and homeostasis are strictly regulated by Zn binding proteins and Zn transporters (Gaither and Eide 2000) which are different from the As transporters. In circulating erythrocytes, major Zn transporter proteins are ZnT1, Zip8, and Zip10 (Ryu et al. 2008), while in leukocytes they are hZnT-1-9 (Overbeck et al. 2008).

Compared to the extent of research investigating MT expression in relation to As cytotoxicity in different cells and organs (Table 2), studies in the hematopoietic system are scarce. In arsenicosis patients, MT mainly MT-1A and 2A transcripts levels in blood and buccal cells were found positively correlated. When compared to healthy subjects, MT levels are significantly lower in arsenicosis patients (Liu et al. 2007). Inorganic As^{III} resistant multiple myeloma (MM) cells have shown an increased expression of the MT-2A, which was found to chelate intracellular inorganic As^{III} (Zhou et al. 2005). In our laboratory, we have found that in vitro human cord blood mononuclear cells (MNC) expressed MT in response to 50 μM of Na-As^{V} (Fig. 1b), albeit, the level of MT expression was higher when MNCs were treated with 100 μM of Zn (Fig. 1a) (Rahman 2001).

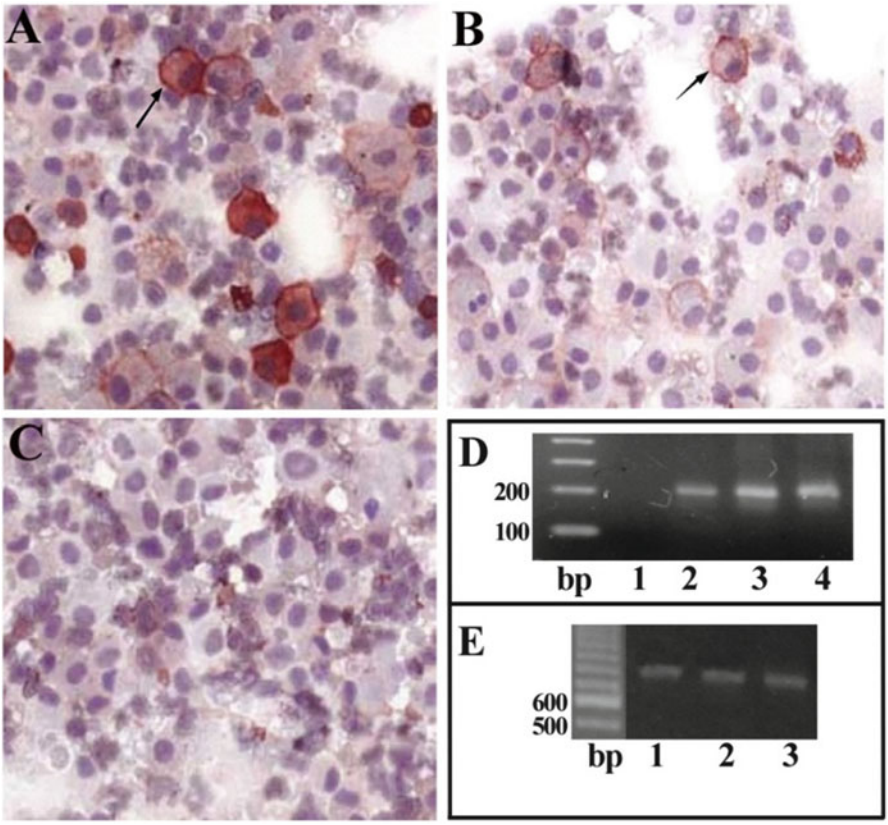

Fig. 1 MT expression in human cord blood MNC in response to in vitro treatment with Na-As^V. MT (*red* at the peri-cytoplasmic spaces) is expressed in MNCs treated with 100 μM of Zn (**a**) and 50 μM of Na-As^V (**b**). Control cultures (**c**), without Zn or As^V, did not show any detectable MT. (**d**) Amplified retro-transcripts of total MT isogenes was similar in MNC cultures treated with 100 μM of Zn (*lane 3*) and 50 μM of As^V (*lane 4*) but higher compared to the control culture (*lane 2*), no band (*lane 1*) was detected after RT-PCR using deionized water instead of mRNA. (**e**) Band intensity for amplified G3PDH retro-transcripts was similar in control (*lane 1*), Na-As^V (*lane 2*) and 100 μM of Zn (*lane 3*) treated cultures. bp, 100 base pair DNA size marker (Rahman 2001)

3 Interaction of As with Blood Proteins

Binding of As to blood proteins such as plasma proteins is a complex phenomenon as it varies from species to species in animals, the route of administration, as well as the proteins involved in the binding. Among different reactive forms of the arsenicals, As^{III} has a high affinity for thiolates of Cys and the imidazolium nitrogen of histidine (His) residues. Typically As^{III} forms three-coordinate trigonal–pyramidal complexes with three Cys in proteins. In hemoglobin (Hb), As^{III} binds with $Cys^{13}\alpha$ and was found responsible for As accumulation in blood (rats fed with As). The

relative reactivity of the Cys in rat Hb was suggested in the decreasing order of: Cys $^{13}\alpha \gg Cys^{111}\alpha > Cys^{104}\alpha$ and $Cys^{13}\alpha \gg Cys^{125}\beta > Cys^{93}\beta$ (Lu et al. 2008). Thus, it is expected that As is bound to Hb through vicinal thiol groups in spleen, bone marrow, plasma and in packed cells.

Besides Hb, As also binds to proteins which have a M_r of 100 kDa, 450 kDa or >2000 kDa in liver cytosol. It has been shown by de novo peptide synthesis that As^{III} -Cys interactions stabilise three-helix bundles found in aqueous solutions (Farrer et al. 2000). When analysing the serum proteins in patients on continuous ambulatory peritoneal dialysis, only the inorganic As species were found to be able to bind to serum proteins, where transferrin is the main carrier (Zhang et al. 1998). It was also shown that, after in vitro incubation in human serum, inorganic As^V binds with serum transferrin (De Kimpe et al. 1993).

Intravenous administration of As^{III}/As^V to mice and rabbits has shown that the percentage of As bound to plasma proteins was 20 % (Vahter and Marafante 1983). When given an intraperitoneal (IP) administration of As^{III} (1 μg of As^{III}/kg mass of the rabbit), the binding of As in plasma proteins increases by about 10 % and 50 % at the 5th and 48th hour (Bertolero et al. 1981). However, prolonged time lapse resulted in gradual decrease of As after IP administration in rabbits. When protein-bound As reached a maximum of 18 % of the total administered As within 20 h, it is then reduced to about 10 % at 120 h (De Kimpe et al. 1996). However, while the percentage of plasma proteins bound As in marmoset monkeys could be as high as 70 % (Vahter et al. 1982), it can also be very low in dogs (Neiger and Osweiler 1989).

4 Interaction of As with Metallothionein

Generally, the two-domain (α and β) MT binds to divalent metals (M) to form two metal–thiolate clusters with stoichiometries of: M_4S_{Cys11} in α-domain and M_3S_{Cys9} in β-domain (Fig. 2a). Using human recombinant MT 1A, Ngu and Stillman (2006) reported that As^{III} binds with stoichiometries of $As_3\ S_{Cys9}$ in both β domain and $As_3\ S_{Cys11}$ in α domain (Fig 2b).

Size exclusion chromatography with inductively coupled plasma mass spectrometry analysis of reaction mixtures between As^{III} and MT clearly demonstrated the formation of complexes of arsenic with MT. Analysis of the complexes using electrospray quadrupole time-of-flight tandem mass spectrometry revealed the detailed binding stoichiometry between As and the 20 Cys residues in the MT molecule (Jiang et al. 2003). Inorganic As^{III} and its two trivalent methylation metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), readily bind with MT (Jiang et al. 2003). Each MT molecule could bind with up to six As^{III}, 10 MMA^{III}, and 20 DMA^{III} molecules, consistent with the coordination chemistry of these arsenicals (Jiang et al. 2003).

The time- and temperature-resolved electrospray ionization mass spectrometry, Ngu et al. (2008) demonstrated that As^{III} binds to MT in a non-cooperative manner

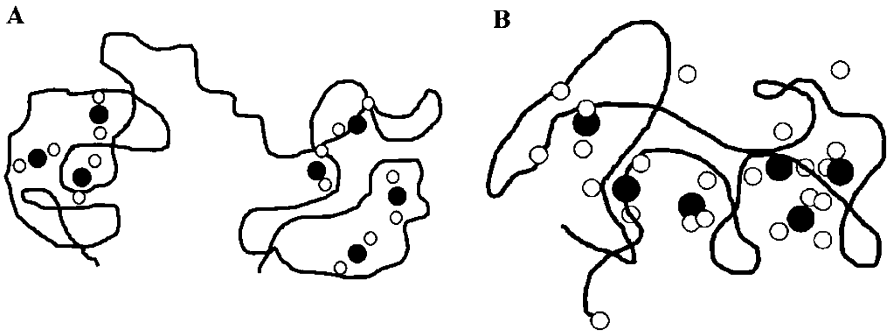

Fig. 2 Metal binding of cysteine (Cys) residues of MT. (**a**) Four divalent metal ions (M^{2+}) such as Cd^{2+} or Zn^{2+} (*filled circles*) generally binds with α-domain while the number of that in β-domain is 3. M^{2+} are bonded with SH (-S-) group of Cys (circles). (**b**) Six As atoms (*filled circles*) are bound to 18 Cys (*white circles*) in MT. Polypeptide backbone is shown in black ribbon with an approximate location of M^{2+}, As and Cys. Figure **a** and **b** are simplified from the corresponding models reported by Ruttkay-Nedecky et al. (2013); and Ngu and Stillman (2006) respectively

involving six sequential reactions in which binding begins with the α-domain followed by the β-domain. Compared to the single domain MT present in cyanobacteria, the two-domain structures allow MT to bind metals faster, and thus make it an efficient metal scavenger (Ngu et al. 2008).

At neutral pH (pH 7), where free As^{III} is not stable, As^{III} that is bound to the recombinant human MT-1A is stable and translocates via protein-protein interactions to other MTs. In vitro studies also confirms that As^{III} transfers from the two-domain β-α-hMT-1A to the isolated apo-β-hMT and apo-α-hMT, where demetallation of the As(6)-βα-hMT occurs in noncooperative manner as apo- and partially-metallated species coexisting in equilibrium conditions (Ngu et al. 2010).

Studies on binding of different metals with MT reveal that mercury (Hg) has the highest affinity for MT while As, Ca and Mo had a limited affinity. When different metals were added to Zn-MT complex, Zn that bound to MT could be replaced in the order of the following affinity: Cd (1.33 μM) > Pb (1.46 μM) > Cu (1.93 μM) > Hg (3.93 μM) > Zn (8.06 μM) > Ag (10.4 μM) > Ni (474 μM) > LCo (880 μM). Al, Cr, Fe, Mg, Mn, Tl and V had no effect on Zn binding even at 1.0 mM (Waalkes et al. 1984). Later Nielson et al. (1985) proposed the metal binding affinity to thiol in the order of: Hg > Cu > Cd > Zn > Ni = Co. Again, Hamer (1986) proposed the affinity in the order of: Hg > Ag > Cu > Cd > Zn.

When partially metallated Cd-MT and Zn-MT were considered the more stable form of metal-apoMT complex, As^{III} transfer at pH 7 is found to be dependent on protein-protein interaction (Ngu et al. 2010). Nonetheless, the cellular redox state as well as the concentration of other biological metal chelators determines the Zn transfer from and to MTs (Jacob et al. 1998). Although initially, Cd or Zn binding to apo-MT is reported to be cooperative (Nielson and Winge 1983), recently, by using recombinant human MT-1A, Sutherland et al. (2012) concluded that the metalation of apo MT occurs in a non-cooperative fashion for both Zn^{2+} and Cd^{2+}.

The binding of As to MT was also reported to be non-cooperative (Ngu et al. 2008). These lines of evidence suggest that even though As toxicity such as increased ROS could induce MT synthesis, the apoprotein might prefer free Zn^{2+} in the cytosol.

Notably, free Zn^{2+} maintains equilibrium in blood through different routes of exchange such as through the erythrocyte membrane permeability. Intracellular Zn^{2+} was found to maintain about 129 μmole/10^{13} erythrocytes while the main component of Zn^{2+} buffering is Hb, with a dissociation constant of about 2×10^{-8} M (Simons 1991).

5 Accumulation and Metabolism of As

Multiple myeloma cells of bone marrow treated with inorganic As^{III} show intracellular biotransformation from As^{III} to As^{V}. Such biological oxidation of As^{III} was described as a protective mechanism of the cell against As cytotoxicity (Falnoga et al. 2007). As^{V} is reduced to As^{III} by CDC25 phosphatases or arsenate reductases (Bhattacharjee et al. 2010). However, biomethylation, particularly the production of As^{III} containing methylated metabolites, is a process that activates As both as a toxin and a carcinogen (Smith et al. 2009). In liver, the intracellular As^{III}-methyltransferase methylates As resulting in formation of both mono and dimethyl As^{V} and As^{III}, and is eventually excreted through bile and urine (Thomas et al. 2007). AQP9 is found to be involved not only in As^{III} uptake from blood to liver, but also in the removal of methylated forms of As down the concentration gradient from hepatocytes to the blood flow to end up in urine (Liu et al. 2006; Carbrey et al. 2009; McDermott et al. 2010). Furthermore, clinical and epidemiological studies have proven that affinity for thiol groups renders As binding to SH moieties of critical proteins like keratin (Lindgren et al. 1982; De Kimpe et al. 1999). Therefore, a variety of skin lesions were linked to As-toxicity (Chen et al. 1988; Brown et al. 1997; Yu et al. 2006).

6 As Cytotoxicity and the Role of Essential Metals

It can be expected that cellular MT induction might act as protective mechanism against As toxicity. This is because production of ROS is one of the major toxic impact of As exposure while MT provides protection against such oxidative stress (Chiaverini and De Ley 2010; Kassim et al. 2013). Induction of MT is achieved through a variety of mechanisms which includes activation of: (1) metal response elements (MRE) by the Zn binding metal-responsive transcription factor (MTF-1) (2) glucocorticoid response elements (GRE) (Kelly et al. 1997), and (3) antioxidant (or electrophile) response element (ARE), in response to the

redox status (Andrews 2000). Zn is also an important regulator of GSH synthesis, where GSH is involved in As excretion (Kala et al. 2000). Zn deficiency is accompanied by the increase in ROS (Kraus et al. 1997; Kojima-Yuasa et al. 2005). In vitro treatment of tARPE-19 cells with 150 μM Zn caused 70 % increase in GSH levels through ARE activated de novo synthesis. ARE activation and GSH synthesis could be inhibited by silencing Nrf2 expression (Ha et al. 2006). Thus, activation of MRE and ARE, by essential nutrients such as Zn might prove beneficial in reducing As toxicity specially to minimize the ROS mediated cytotoxicity.

Cellular metabolism consistently generates ROS, where intracellular GPx plays an important role to reduce ROS such as H_2O_2 to water, hence limiting the harmful effects of the ROS. GPx is a selenocysteine-containing enzyme, expression of which is strictly regulated by the supply of Se and selenocysteine (reviewed by Lubos et al. 2011). Therefore, the in vivo acceptable range of Se supplement to induce antioxidant mechanisms such as GPx and GSH might be beneficial in the reduction of As toxicity, which is mostly linked with ROS. Notably, several lines of evidence have proven the beneficial impact of essential metals such as Zn and Se supplement against metal toxicity, such as cadmium and chromium (Table 3). Cd toxicity on sperm motility and the testicular antioxidant status could be restored by Se and Zn supplement (Saïd et al. 2010). Again Se supplements improved renal toxicity biomarkers' levels and antioxidant enzyme activities in $K_2Cr_2O_7$ administered renal damages (Soudani et al. 2010). Similarly, co-administration of Se with $K_2Cr_2O_7$ restored hematological dysfunction related to the Cr exposure to near-normal values (Soudani et al. 2011). Furthermore, based on a number of in vivo and in vitro studies, McCarty (2012) proposed Zn supplement to ameliorate pathogenic impact of Cd toxicity as Zn is known to have protective anti-inflammatory, antioxidant, and immunosupportive effects. Therefore, it is not unexpected that Zn and Se supplement could be beneficial to minimize cytotoxicity exerted by As (Fig. 3).

7 Conclusion

As, commonly known as a metalloid, has been used to treat cancer, such as the acute promyelocytic leukaemia (Dilda and Hogg 2007), infectious diseases (Frézard and Demicheli 2010) and sleeping sicknesses (Chappuis 2007), the same metalloid is also able to cause cancer and damage the liver and the kidneys. As a means of treatment to As induced damages, essential metals such as Zn and Se could be beneficial, as these metals can induce both intracellular MT and antioxidant mechanisms.

Table 3 Essential metal supplement to treat toxic metal exposure

Toxic metal exposure	Toxic impact	Essential metal supplement	Beneficial impact	Experimental model (Reference)
Cd (Male rat)		Se and Zn	Restoration of the sperm motility and the testicular antioxidant status	In vivo (Rat) (Saïd et al. 2010)
Cd toxicity		Zn	Protective anti-inflammatory, antioxidant, and immunosupportive effects	Review based Hypothesis founded on in vivo and in vitro studies. (McCarty 2012)
$K_2Cr_2O_7$ for 21 days	Renal damages with a significant increase in kidney malondialdehyde, superoxide dismutase, plasma creatinine, and uric acid levels, while catalase, glutathione peroxidase, non-protein thiol, Metallothionein and plasma urea levels decreased	Se	Improved malondialdehyde, renal biomarkers levels and antioxidant enzyme activities. Kidney histological studies confirmed biochemical parameters and the beneficial role of selenium	In vivo (Rat) (Soudani et al. 2010)
$K_2Cr_2O_7$ (700 ppm), in drinking water	Increase of malondialdehyde and protein carbonyl levels and a decrease of sulfhydryl content, glutathione, non-protein thiol, and vitamin C levels. A decrease of enzyme activities like catalase, glutathione peroxidase, and superoxide dismutase activities was also noted in erythrocytes	Se (0.5 mg/kg of diet) for 3 weeks	Co-administration of Se with $K_2Cr_2O_7$ restored the parameters cited above to near-normal values.	In vivo (Rat) (Soudani et al. 2011)

8 Summary

Millions of people are exposed to a toxic level of arsenic (As). Oxidative stress due to As is evident in organs such as the skin, liver, kidneys and lungs. Several intracellular antioxidant defense mechanisms including glutathione (GSH) and metallothionein (MT) have been shown to minimize As cytotoxicity. The current

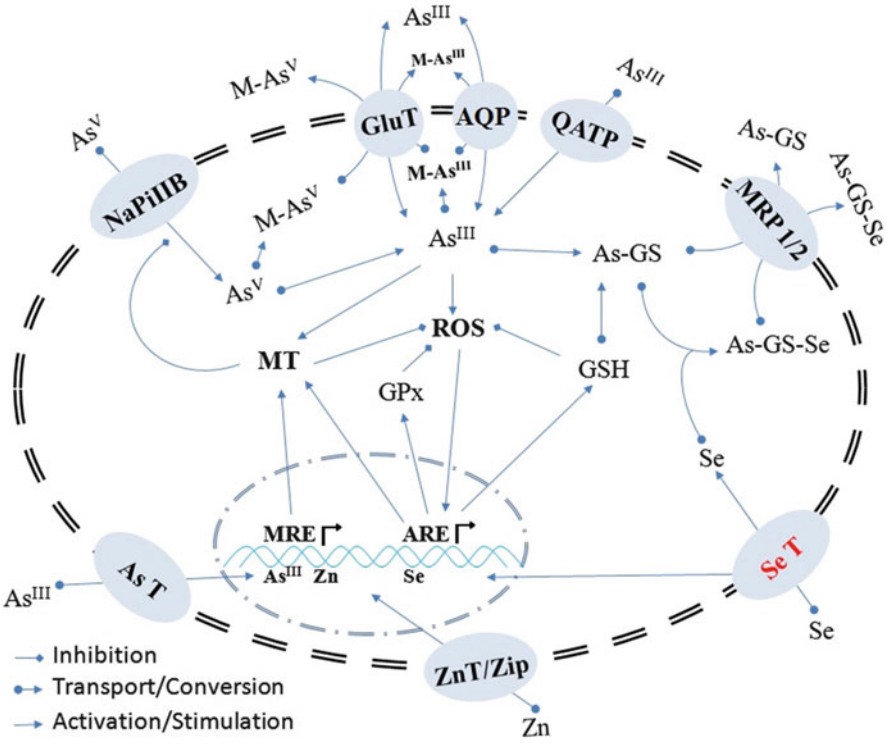

Fig. 3 Intracellular As toxicity and its possible remediation. Once internalized, As^{III} and As^{V} via As^{III} will generate reactive oxygen species (ROS). As^{III} and As^{V} via As^{III} can also induce metallothionein (MT) which in turn will reduce ROS and block intracellular transport of MT respectively. If given Zn and Se supplement, As^{III} inducible ROS can be further reduced by induction of additional MT and glutathione peroxidase (GPx) or glutathione (GSH). [*AQP* aquaglyceroporins, *ARE* antioxidant response elements, *AsT* As transporter, *GluT* glucose permease, *M-As* methylated As, *MRE* metal response elements, *MRP* multidrug resistant protein, *NaPiIIb* sodium-phosphate cotransporter, *QATP* organic anion transporting polypeptides, *ZnT/ZIP* Zn transporter proteins]

review summarizes and the involvement of MT as an intracellular defense mechanism against As cytotoxicity, mostly in blood. Zinc (Zn) and selenium (Se) supplements are also proposed as a possible remediation of As cytotoxicity. In vivo and in vitro studies on As toxicity were reviewed to summarize cytotoxic mechanisms of As. Intracellular antioxidant defense mechanisms of MT are linked in relation to As cytotoxicity. In addition, in vitro potential of pentavalent inorganic As to induce MT biosynthesis was evaluated in human peripheral blood mononuclear cells. Arsenic uses a different route, compared to major metal MT inducers such as Zn, to enter/exit blood cells. However, a number of in vivo and in vitro studies showed that upregulated MT biosynthesis in blood components are related to toxic levels of As. Despite the cysteine residues in MT that aid to bind As, MT is

not the preferred binding protein for As. Nonetheless, intracellular oxidative stress due to As toxicity can be minimized, if not eliminated, by MT. Thus MT induction by essential metals such as Zn and Se supplementation could be beneficial to fight against the global As toxicity.

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Mobility and Fate of Pollutants in the Aquifer System of the Northwestern Suez Gulf, Egypt

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1 Introduction

The Egyptian government has established huge projects along the northwestern Suez Gulf region to plan for future economic development. Therefore, northwestern Suez Gulf is the essential investment area and a significant source of the Egyptian national income. These investments include projects such as oil refineries, fertilizer and chemical industry, power stations and the construction of huge commercial harbours near to the industrial zone. Also, 450 newly developed industrial projects are planned in this region, e.g. steel factory, power station, pipelines, marinas, touristic villages, vegetable and edible oil industries, ceramics, shipyard, cement, fiberglass... etc. (El-Moselhy and Gabal 2004). The rapid growth of industrialization has significantly changed the ecosystem of this region. The Suez Gulf is considered the heavily polluted area in the Red Sea and Suez Bay is the worst region (e.g., Khaled et al. 2004; El-Sikaily et al. 2003, 2004; El-Nemr et al. 2004a, b). The utilization of resources in the study area and their future development mainly depend on the creation of actual hazardous pollutants control programs. These pollutants increase day by day if steps are not taken for environmental protection during the northwestern Suez Gulf development. It is accepted that environmental quality control and pollution reduction activities are components of any economic development and resources utilization programs.

The main objective of this study is to identify the existing levels of anthropogenic contamination in the northwestern Suez Gulf region, as a function of the location and time. Also, to establish the basis for future monitoring strategies and a provide baseline of considerable amount of available data that will allow assessing and evaluation of future changes, in an area that has not been extensively surveyed before.

1.1 Background

The northwestern Suez Gulf is rapidly becoming among the largest coastal oil refineries areas. On one hand, the anthropogenic origins of petroleum pollution have led to an interest in their distribution and fate in the environment during the

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previous decades. So, extensive studies mainly addressing the marine environments were resulted. On the other hand, no studies concerned with the movement of contaminants in the subsurface and aquifers have been carried out. Therefore, the spread and movement of pollutants in subsurface is a subject that deserves discussion in the study area. Hanna (1983) indicated that the level of total dissolved oil in the Egyptian Red Sea coastline was not too hazardous, these results were in line with the study given by Cambridge University (1981). Hanna (1989) detected different values of heavy metals such as Pb in the Red Sea fishes and found that it was falling far below the tolerance levels recommended by WHO (1973) for human consumption. Hanna (1992) found that the average of heavy metals content in the sediments of the Red Sea have increased during 50 years from 1934 to 1984 because of environmental pollution. For example Pb and Cd average concentrations were 5 and 3 times, respectively, greater in 1984 sediments than in 1934 sediments. He indicated that the increase in both Pb and Cd concentrations in sediments of the Red Sea may be attributed to oil pollution. Abd El-Moniem et al. (1994) detected some trace metals (Cd, Pb, Cu, and Zn) in organs of three fish species collected from the Suez Gulf, and showed that these metals accumulated in a great extent especially Pb. They conclude that Suez Bay is influenced by industrial effluent especially that originated from oil refineries. Also, they mentioned that these internal organs should not be used as fish meals and other nutrition purposes. Consequently, Hanna (1995) applied the 'Vulnerability Index' (VI) to evaluate and monitor the consequence of the oil spill and pollution on the Egyptian Red Sea coast. VI was rating of 1–10 on an arbitrary scale for various eco-systems, where 10 represents total cover or severe pollution. The geomorphological/VI results show that the northwestern Suez Gulf area has high vulnerability (7–10 on the VI) to oil spill damage.

Hamed and Said (2000) stated that the Suez Bay area represented a eutrophic region (high productivity). This is owing to industrial activities sewage that spread along the western coast of the Bay. They noticed that the northward Suez Gulf is influenced by the disposal of mainly acidic sewage and industrial effluents, in addition to oil refineries effluents distributed in the Suez Bay area. Also, they detected a concentration of heavy petroleum fractions in the range of 4.58–4.86 μg/L in water samples that were collected from the northern Suez Gulf (Suez Bay). They concluded that the water quality of the Suez Bay is gradually deteriorating as a result of such pollution. El-Sikaily et al. (2003) mentioned that the industrial development of the Red Sea coast caused increased concentrations of pollutants day by day. Especially by polycyclic aromatic hydrocarbons (PAHs) that are natural constituents representing about 20 % of total hydrocarbons in crude oil and contribute substantially to the severe toxicity of all the petroleum compounds. Also, they stated that a mixed petroleum product that contains a broad spectrum of hydrocarbon classes is released to the marine environment. It probably enters the food chain, harm a variety of biological processes and can be a potent cell mutagen and carcinogen, when ingested by marine animals. So, it can form metabolites that are active carcinogens. They recognized that coral reefs present on the Egyptian

Red Sea coast, are the most deteriorated ecosystems and considered to be in critical status in many places.

Shams El-Din et al. (2004) mentioned that the Suez Bay is under environmental stress of industrial wastes discharges such as from oil refineries. These wastes and effluents are potentially increasing year after year through intense expansion of the several types of activities along the western bay coast. Also, they indicated that all these discharged pollutants accumulating year after year till will reach to a certain level that converts the bay and the Suez Gulf to a heavily polluted environment. They concluded that the subjection to different types of pollution impacts the distribution and biodiversity of some marine organisms as well as the environmental conditions prevailing in Suez Bay. El-Nemr et al. (2004a) indicated that some marine organisms at northwestern Suez Gulf (Suez to Ain Sukhna) are polluted by aliphatics and aromatic hydrocarbons (1890–4666 ng g^{-1} the concentration in muscles) when compared with the other Red Sea coastal areas. El-Nemr et al. (2004b) detected chlorinated organic compounds that have a wide range of industrial applications in the coral reef skeleton investigated at many sites in the Egyptian Red Sea coastal marine environment. The highest concentration measured for polychlorinated biphenyls (PCBs) was 48.3 ng g^{-1}. They stated that these synthetic organochlorines cause a serious threat to the long-term health of the marine environment for many years. This is because of their strong accumulation in lipid tissues of marine biota and their high toxicity for marine organisms, and also because of the slow degradation of several members of chlorinated organics group.

El-Sikaily et al. (2004) stated that the analyses of some heavy metals such as Cd, Co, Pb and Ni showed higher average concentrations for some costal marine organisms samples collected from the Red Sea. In the Suez Gulf recorded concentrations of these anthropogenic inputs of heavy metals amount to 0.53–0.75, 1.64–2.37, 1.36–1.43 and 2.06–4.66 μg/g wet weight for Cd, Co, Pb and Ni, respectively. El-Moselhy and Gabal (2004) determined Cd, Pb, Cu and Zn in water, sediments, and marine organisms (gastropod and green algae) collected from the northwestern Gulf of Suez side during the period February 1993–January 1994. The highest values of these metals were found at locations influenced by various pollution sources such as industrial drains. In contrast, the lowest concentrations were observed faraway from pollution source. The metal concentrations found in coastal water were 0.15–0.18 μg/L for Cd, 1.84–2.57 μg/L for Pb, 1.16–5.33 μg/L for Cu, and 8.13–23.24 μg/L for Zn. The concentrations found in sediments amount to 2.26–4.40 μg/g for Cd, 13.90–28.34 μg/g for Pb, 1.84–10.25 μg/g for Cu, and 4.26–23.68 μg/g for Zn. The concentrations of Cu, Zn, Cd and Pb in gastropods are 28.19–72.04 and 60.24–108.74 μg/g, 0.12–0.41, and 1.15–4.00 μg/g, respectively. The concentrations of Cd, Pb, Cu and Zn in green algae were 0.69–1.48 μg/g, 3.87–23.29 μg/g, 8.88–13.27 μg/g and 15.23–49.65 μg/g, respectively. They concluded that sediment is a good indicator for Pb pollution, and green algae are a good indicator for the toxic metals, Cd and Pb.

Ibrahim (2004) classified northwestern Suez Gulf as a highly contaminated area with PAHs, with the levels of total exceeding 1000 μg kg^{-1} (levels in the sediment ranged from 210 to 23696 μg kg^{-1}), and indicated that coastal Suez Gulf oil

pollution is a serious problem. Khaled et al. (2004) detected organochlorine pollutants in some marine organisms collected from different locations along the Egyptian Red Sea coast. They found high concentrations of PCBs recorded in northwestern Suez Gulf (Suez and Suez Bay) with values in the range of 28.66–66.44 ng/g of wet weight in mussel samples. Also, they indicated that the levels of PCBs contamination in the investigated mussels were low to moderate. El-Shenawy and Farag (2005) investigated some bacterial groups in surface coastal waters samples collected from Suez Gulf and indicated that the water quality was generally affected by anthropogenic influences. Said and Hamed (2006) stated that the northern part of the Suez Gulf region environment has significantly changed because of rapid growth of industrialization along the Red Sea coast. That region receives considerable pollution discharged from oil refineries to the Suez Gulf. They mentioned that the average total concentrations of PAHs in the water collected from Suez Gulf recorded 38.717 μg/L.

El-Nemr et al. (2006a) detected hydrocarbons contaminations in all sediment samples that were collected from the Suez Gulf coastal area. PAHs in these sediments ranged from 158 to 10463 ng g^{-1}, the aliphatic hydrocarbons concentrations varied from 0.52 to 88.38 ng g^{-1}. El-Nemr et al. (2006b) stated that Suez Gulf sediment contains extremely high concentrations of heavy metals (Cd and Pb), indicating a high new input of these metals to the Suez Gulf. Hamed and Emara (2006) determined the levels of the heavy metals Cu, Zn, Pb, Cd, Cr, Ni, Mn and Fe in coastal water, sediments and some marine organisms in samples collected from different locations in the western coast of Suez Gulf. The concentrations of these heavy metals in water reached 3.37–4.78, 18.83–21.46, 2.75–3.17, 0.22–0.27, 0.99–1.21, 2.69–3.65, 3.75–4.56 and 23.82–32.78 μg L^{-1} for Cu, Zn, Pb, Cd, Cr, Ni, Mn and Fe, respectively. The corresponding concentration values in the sediments were 8.65–12.16, 51.78–58.06, 36.52–42.15, 3.23–3.98, 9.03–12.75, 34.31–49.63, 3.28–4.56 and 64.20–70.22 μg g^{-1} for Cu, Zn, Pb, Cd, Cr, Ni, Mn and Fe, respectively. The highest accumulated metals in marine organisms were 37.81–70.91, 2.37–2.13, 14.57–9.88 μg g^{-1} and 2.74–2.94 mg g^{-1} for Pb, Cd, Ni and Fe, respectively. They indicated that the worst polluted region in the Suez Gulf was Suez Bay, owing to the direct influence of many pollution sources on the western coastal area of the Gulf. The Suez Bay is subjected to pollution from land based activities and dense industrial waste disposal come from oil refineries. Rushdi et al. (2009) stated that sediment samples collected from the coastal zone of the Suez Gulf contain a variety of anthropogenic organic compounds from petroleum activities (discharges from refineries and petrochemical plants).

Abdel-Hamid et al. (2011) detected elevated levels of heavy metals such as Pb, Co, Cd, Ni and Fe in the seawater, sediments and corals at reef sites in the northern Red Sea. The trace metals concentrations in seawater were 0.80, 0.52, 0.43, 5.80, 28.35 μg L^{-1} for Pb, Co, Cd, Ni and Fe, respectively, whereas in sediment concentrations were 12.64, 13.29, 4.30, 18.00, 1285.00 μg g^{-1} for Pb, Co, Cd, Ni and Fe, respectively. However, the trace metals concentrations in coral species were in the range of 6.89–16.36, 1.36–7.10, 0.89–9.59, 186–4.20 and 27.31–321.54 μg g^{-1} for Pb, Co, Cd, Ni and Fe, respectively. They indicated that

the considerably elevated levels of heavy metals in seawater, sediments and corals collected from reef sites resulted from increased environmental contamination, because of anthropogenic sources of pollution. They mentioned that some kinds of corals displayed the highest concentration of Pb of the surveyed heavy metals at Suez Gulf. Also, they demonstrated that the percentage cover of dead corals were significantly higher as the concentrations of heavy metals increased.

El-saied et al. (2011) indicated that the Suez marine environment is suffering from varieties of anthropogenic pollutions. They detected five Suez signal-related genes cassette-encoded associated with marine oil-polluted sediments extracted from Suez Bay. Also, they mentioned that this is the first report on the existence of bleomycin (to survive in these niches, each bacterium must develop a resistance to myriad natural antibiotics (D'Costa et al. 2006)) resistance genes in marine sediments. This enzyme specifically acts on halide bonds in carbonhalide compounds and participates in degradation of both gamma-hexachlorocyclohexane and 1, 2-dichloroethane, a toxic long-lived organochlorine, that has been detected in Suez Bay. Moreover, appearance of gene cassettes that encoded haloacid dehalogenases probably correlated with the stress of high concentrations of PAHs in Suez sediment El-Nemr et al. (2006a).

Shreadah et al. (2011) detected some organometallic compounds (organotin and organolead), that have different toxicological behavior compared to that of inorganic compounds of the respective elements, in coastal sediment samples of the Suez Gulf. They mentioned that industrial discharge is the main sources of pollution by these compounds in Suez Gulf. In addition, they stated that water circulation is important for the transport mechanism of these organometallic compounds, where the persistent anticlockwise circulation in the Suez Bay causes more pollution in the western side.

Belal and Ghobashy (2012) stated that some marine organisms recorded high values of heavy metals such as Pb, and can be used as biomonitor to these elements in seawater especially in all the Suez Bay. El-Nemr et al. (2013) detected persistent organic pollutants in surficial sediment (fine to coarse sand) samples along the Egyptian Red Sea coast. They attributed the presence of some organic contaminants such as PCBs to some industrial activities. Northwestern Suez Gulf polluted sediments recorded PCBs concentrations that varied from 0.91 ng/g at Suez in the north to 1.08 ng/g at Ain Sukhna in the south.

Zawrah et al. (2013a, b, c) detected a concentrations of petroleum paraffines (17.53–7.24 wt %), PAHs (107.23–10.36 mg/kg) and Pb (68.74–399.95 mg/kg), in sediments collected from a petroleum refinery located at northwestern Suez Gulf coastal plain. They mentioned that the petroleum refineries are the foremost continuous source of pollution by both heavy metals and organic contaminants in northwestern Suez Gulf region.

Ali et al. (2014) found concentrations of PAHs (399.616 up to 67631.779 ng/g) especially six rings members in fish samples collected along north Suez Gulf, that are high enough to cause lethal toxicity effect by accumulation.

Farid et al. (2014) determined petroleum pollutants in Suez Bay coastal waters with concentrations amounting to 3.88–37.69 mg/ml. They indicated that the Suez

Gulf suffers from extensive chronic pollution especially crude oil inputs from different sources. Ahmed et al. (2014) detected petroleum hydrocarbon concentrations of 987.439–5007.2 ng/g wet weights, in some fish species collected from Suez Gulf. They indicated that the edible tissues were extremely toxic especially in Suez Bay area, because of ingestion of contaminated materials by these fish. El-saied (2014) recorded gene modification in the DNA data base that represented the first discovery species in the Suez Gulf (Suez Bay) in some marine microorganisms found in the sediments. This author also, stated that these new genera are widely distributed in polluted aquatic environments, controlled by the anoxic condition that is characterized by heavy hydrocarbon pollution, where the oxygen level is zero. He concluded that more metagenomic survey should be done, based on functional genes to understand the ecological role of these microorganisms within this polluted environment. Snousy et al. (2015) detected diverse concentrations of PAHs in sediments samples collected randomly from an industrial site adjacent to Suez Bay, ranging between 10.36 to 107.23 mg/kg. They indicated that the majority of PAHs in these samples is heavy fractions that consist of 4 and 6 rings.

2 Study Area Description (Geology and Hydrogeology)

Environmental professionals have focused on risk-based approaches for remediation of polluted sites. This, to help preserve green lands (pristine land) from pollution caused by industrial development and also to provide opportunities for economic growth. This part includes reviewing the relationship between geological-hydrological and environment pollution investigations conducted on a broad scale. To ensure the sustainable development in this area, an assessment of water resources management (run off, flash flooding potential), should be carried out to get optimum utilization of every possible drop of water and to avoid the elevation of pollutants levels.

The geomorphological/vulnerability index results show that the Egyptian Red Sea coastal environments have medium to high vulnerability i.e. high biological susceptibility to immediate and medium term oil spill damage. Coarse-grained sandy and gravel beaches that are subjected to oil penetration and burial are assigned intermediate index values of 4–7. The 'Vulnerability Index' proposed by Hanna (1995), ranks coastal environments on a scale of 1–10 based on the degree of susceptibility to oil deposition and residence time. This vulnerability index assists oil-spill planners and decision makers in formulating a first line of defense against influence by oil and designing of the cleanup processes along any coastline, since it locates the areas of the highest and the lowest geologic and ecologic sensitivity of the shoreline. Oil spill from oil production in the north western part of the Suez Gulf (see Fig. 1) was the worst affected part by oil of the areas surveyed from the shoreline between Suez and Ghardaqa. So, the north western part of the Suez Gulf region has chronic oil pollution because of oil pollution from the continuous activities of oil productions as mentioned by (Hanna 1995).

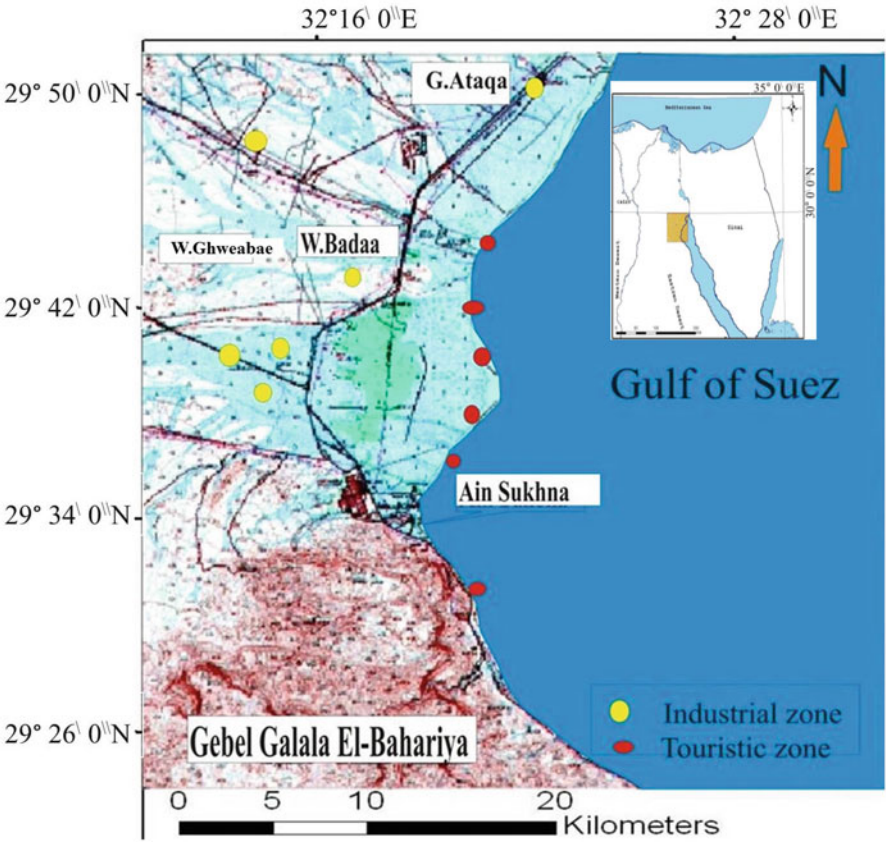

Fig. 1 Location map shows some activities in the study area (after Mohamed 2010, with modifications)

The northwestern Suez Gulf climatic conditions prevailing in the area are typically of arid provinces of North Africa. A hot summer and a warm rainy winter characterize the climatic conditions in the area (Soliman 2010). The area receives moderate amounts of rainfall precipitation ranging from 60 to 75 mm/year (Sultan et al. 2011). The area is characterized by a highly complex and varied relief whose detailed forms reflect the rock composition and the effect of structural processes. The sedimentary rocks which comprises the northwestern Suez Gulf area ranging in age from Jurassic to Recent with some doleritic intrusions. The distribution of these rocks was controlled by earth movements accompanied by intense faulting that resulted in rising the main mountain blocks (Usama 2001).

The Gulf of Suez, in general, is one of the intensively faulted areas in Egypt (faults are the main subsurface structural features present in the area while folding has a minor role). The origin of faults is the tensional forces (Said 1962). Minor faults have a general trend of SE-NW trend and dip towards the (NE) at angles

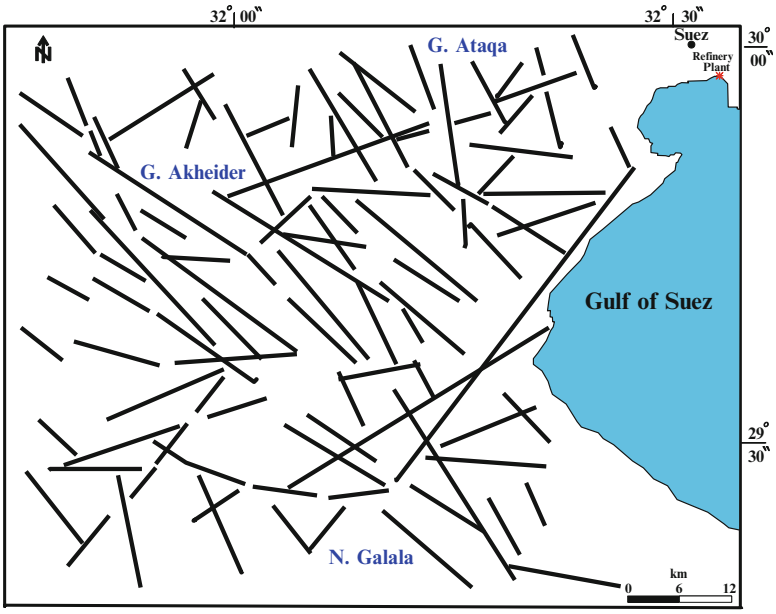

Fig. 2 Lineament map of the area between G. Ataqa and northern Galala (after EL-Shazely 1977)

ranging from 64° to 80° (Fig. 2). In contradiction with other Bathonian exposures at Khshm El-Galala, Ras El Abd and wadi Um Lug, this exposure is due mainly to faulting accompanied by sliding. Hence the exposed Bathonian rocks are overlain westwards by younger rocks. It is generally noticed that the approximately E-W and SE-NW faults are the predominant structural lines in the area (Usama 2001).

The physiographic units present in the Gulf of Suez area are closely connected to the complicated subsurface tectonic features, the strong geological variations, the high relief and the paleo-wet climates. The area is representing a portion of the complex hydrological formations dominating the northern part of the Eastern desert. The groundwater aquifers of the area occur under different conditions reflecting the remarkable structural, lithologic and topographic variations. Groundwater is representing the main source of water in the desert lands that is used mainly in agriculture, and industrial uses. Many wells of available depth have been drilled by companies in the desert areas to extract water for treatment and to be used in industry.

The northwestern Suez Gulf aquifer extends into Sinai and comprises two main components. The groundwater aquifer in the desert area is represented by a succession of sand silt and gravels belonging to Quaternary sediments. Based on the stratigraphic sequence dominating the investigation area, the water-bearing formation is classified into Tertiary (Miocene fissured carbonate and Pliocene deposits) and Quaternary alluvial aquifers from older to younger as shown in Fig. 3. The Quaternary deposits mainly form and cover the target site surface and the most surrounding area. Also, the Quaternary sediments of sand and gravel represent the main aquifer in the northwestern part of Suez Gulf area. The Quaternary aquifer

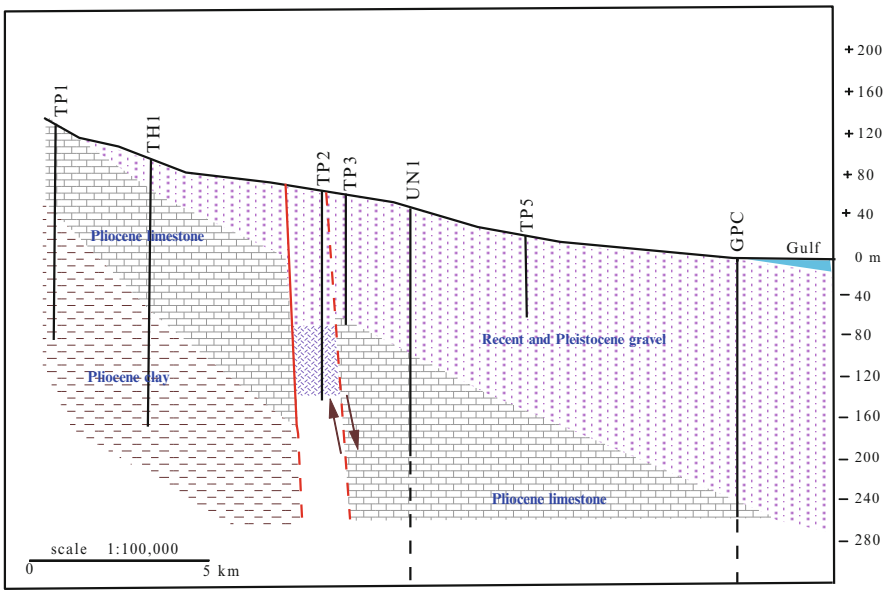

Fig. 3 Lithological cross section for some drilled wells in the area (REGWA 1979)

has developed at the deltaic areas of the main wadis that cross the coastal plains. The water exists under phreatic conditions nearly at sea level, and its salinity is about 1200–7000 ppm. The Tertiary aquifer has developed at great depths, and its salinity reaches up to 14,000 ppm (Mohamed 2010).

3 Study Approach and Discussion

An accurate prediction of aquifers and risk management intensity in the region is highly required and a prioritization scheme for pollution control and environmental protection is essential for the sustainability of the development. Restrictions and monitoring the following consecutive releases of pollutants is a key step towards developing actual approaches to contaminants removal. Also, identifying the pollutants sources in space and time is important in determining the resulting spatial and temporal distribution of concentrations within the groundwater system. In all cases, environmental considerations must be strictly observed in this dynamic economical area.

This section deals with assessing the risks resulting from the contaminants spread that probably threaten groundwater basins as detected in some drilled wells located at northwestern Suez Gulf and polluted by heavy metal specially Pb (Mohamed 2010), as shown in Fig. 4 and listed in Table 1. It is also clear that contaminants in coastal water have been poorly studied relatively to freshwater

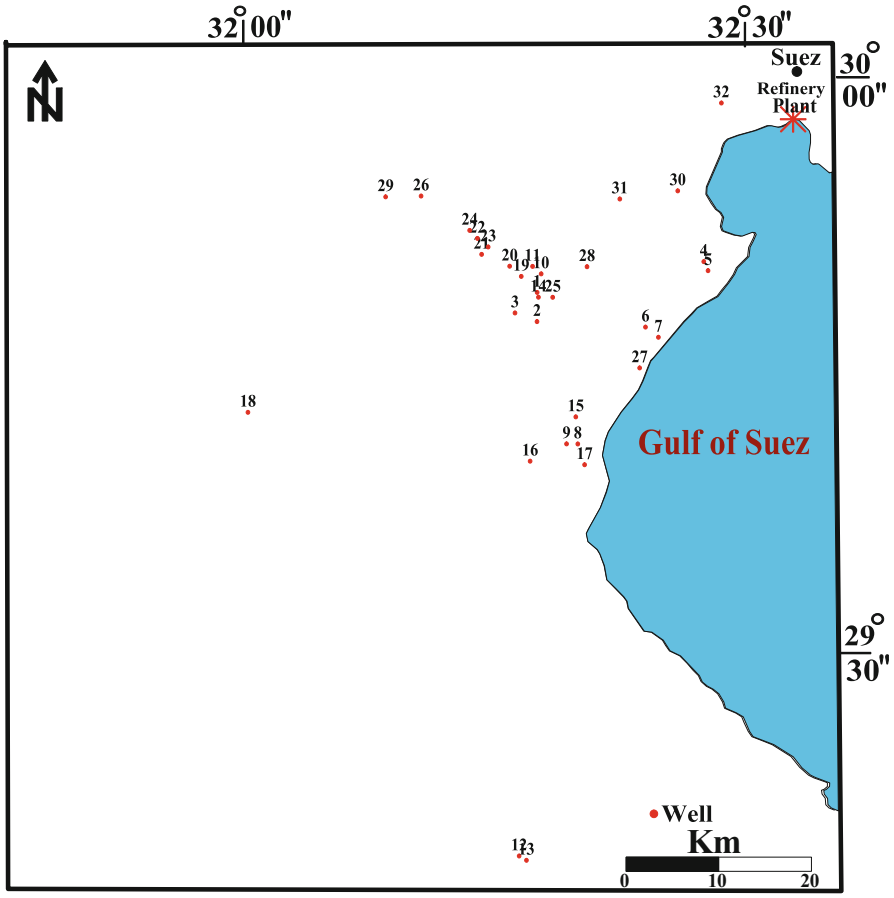

Fig. 4 Location map of some drilled wells

environments. These contaminants cause direct toxic effects when released into aquatic environments as mentioned in the previous studies (Section "Background"). The scenarios that possibly explain the spread of pollutants (organics and heavy metals) depend on the entering of these pollutants into the surface and ground waters environment, directly because of spills or leakage from costal petroleum refinery or other industrial facilities. This spread is due to the movement of free phase and leaching of residual or adsorbed phases that usually represents a long-term contamination source for soil and groundwater (Mackay 1985). Sediments frequently release adsorbed contaminants back to the pore water, and may cause to aquatic life as well as human beings.

As mentioned previously, the Quaternary sediments, which are predominantly sand and gravel represents the main deposits in the northwestern Suez Gulf. These sediments do not have a high adsorptive capacity. Law (1981) and Zanardi et al. (1999) mentioned that the gravely and sandy soils are most vulnerable. The

Table 1 Location and hydrogeological data of some drilled wells (Dabash 2008; Mohamed 2010)

Serial no.	Age	Well name	Location		Elevation (m) as sea level	Total depth (m) as drilling	Water depth (m)	Pb concentration (mg/L)
			Longitude	Latitude				
1	Quaternary	El Misrh-1	32° 17\ 20\\	29° 42\ 30\\	33.60	135	1.30	0.06
2		El Misrh-4	32° 16\ 20\\	29° 41\ 35\\	39.40	140	1.50	0.02
3		El Misrh-5	32° 15\ 29\\	29° 41\ 51\\	56.30	130	2.70	0.008
4		El Hndsi-1	32° 22\ 45\\	29° 43\ 30\\	13.25	60	1.90	0.05
5		El Hndsi-2	32° 22\ 55\\	29° 43\ 13\\	13.00	60	2.40	0.006
6		El Hndsi-3	32° 20\ 31\\	29° 41\ 26\\	4.23	60	1.60	0.01
7		El Hndsi-4	32° 21\ 01\\	29° 41\ 07\\	4.25	60	1.80	0.006
8		Somid-1	32° 17\ 56\\	29° 37\ 45\\	21.68	65	2.30	0.04
9		Somid-2	32° 17\ 30\\	29° 37\ 45\\	25.10	57	2.60	0.05
10		S.Cement 6	32° 16\ 29\\	29° 43\ 05\\	50.50	175	1.80	0.009
11		Tp2	32° 16\ 09\\	29° 43\ 19\\	50.70	170	2.90	0.14
12		Tp3	32° 15\ 46\\	29° 24\ 48\\	53.00	145	3.70	0.11
13		Tp4	32° 16\ 03\\	29° 24\ 40\\	50.00	140	4.60	0.08
14		Tp5	32° 16\ 23\\	29° 42\ 21\\	44.80	144	3.90	0.04
15		UNI	32° 17\ 51\\	29° 38\ 36\\	23.31	57	1.60	0.11
16		Ghouber-1	32° 16\ 06\\	29° 37\ 12\\	37.96	90	2.50	0.18
17		Elasmda-1	32° 18\ 12\\	29° 37\ 06\\	20.20	70	2.40	0.01
18		Geol.Sur.2	32° 05\ 13\\	29° 38\ 40\\	41.80	190	3.40	0.03
19		S.Cement5	32° 15\ 34\\	29° 43\ 02\\	54.00	122	5.60	0.32
20		S.Cement 8	32° 15\ 16\\	29° 43\ 19\\	55.60	250	4.20	0.27
21		S.Cement 2	32° 14\ 11\\	29° 43\ 42\\	58.00	110	4.80	0.14
22		S.Cement 3	32° 14\ 01\\	29° 44\ 11\\	62.30	210	5.30	0.22
23		S.Cement 4	32° 14\ 26\\	29° 43\ 55\\	64.60	179	6.70	0.17
24		S.Cement 2	32° 13\ 43\\	29° 44\ 26\\	65.20	250	4.90	0.24
25		S.Cement11	32° 16\ 56\\	29° 42\ 31\\	33.00	143	5.10	0.16

26	Pliocene	S.cement h	32° 11\ 50\\	29° 45\ 30\\	111.60	110	20.00	0.18
27		El Nagma	32° 20\ 18\\	29° 40\ 09\\	14.00	62.5	18.00	0.19
28	Miocene	Geol. Sur.1	32° 18\ 15\\	29° 43\ 19\\	26.12	180	24.00	0.13
29		Geol. Sur.4	32° 10\ 28\\	29° 45\ 28\\	126.00	250	45.00	0.17
30		Geol. Sur.6	32° 21\ 44\\	29° 45\ 43\\	56.00	110	42.00	0.02
31		Geol. Sur.5	32° 19\ 30\\	29° 45\ 27\\	56.00	180	64.00	0.08
32		Geol. Sur.7	32° 23\ 24\\	29° 48\ 29\\	67.00	60	52.00	0.12

pollution of groundwater becomes a question of hours. The sandy soils are vulnerable and critical in accidental leaks, and seepage velocity is ranging from 13.2 to 2.8 m/h for gravely sand sediment (Halmemies et al. 2003). Since organic pollutants strongly adsorb in soils, especially onto *terrestrial colloids*, also, many organic compounds have low solubility in water, and leach for those (sparingly soluble in water) from the soil over a longer period of time resulting in long-lasting environmental effects.

Organic pollutions, certainly, are widely dispersed because of high pumping or withdrawal rate of groundwater (Zawrah et al. 2014). El-Osta et al. (2010) stated that the extraction rate is about 3000 m^3/day from the Quaternary aquifer, this allows organic pollution to circulate within a large fraction of the porous medium. No advanced waste treatment was employed to remove contaminants from these continuous point sources used by industry. Contaminant concentrations in adjacent coastal areas were rapidly increased because of growing wastewater discharges. Dabash (2008) mentioned that about 90 % of water consumption in northwestern Suez Gulf is extracted from shallow Quaternary sandy aquifer. This has resulted in fluctuation of organic contaminants in the whole Groundwater basin and may lead to leaching of organics. Hence, it constitutes a high pollution risk for adjacent areas. Also, industrial effluents are seeping into Suez Gulf water nearby.

Leachability of organic pollution has increased because of intensive periods of Gulf water regressions and transgressions. This affects the aquifer dependability as a continuous source of water in northwestern Suez Gulf, and deteriorates water quality.

Heavy metal pollution arising directly from anthropogenic activities (industrial wastes) is the contamination in this area. Pb is the most abundant heavy metal contaminant in northwestern Suez Gulf ecosystems. It is transported mainly in colloidal forms in the subsurface system. Colloid-facilitated transport is a major pathway for strongly adsorbed contaminants, operationally defined as particles in the range of 1 nm to 1 μm in size. Colloids in the subsurface are generally minerals, organics, or biological origin. Colloidal fractions in the soil–subsurface solid phase interact with heavy metals, inducing an increase in their mobility. The transport of colloid-bound Pb increases confirming the pathway of heavy metal and metalloid colloid-facilitated transport (Berkowitz et al. 2014). High Pb concentrations have been observed within northwestern Suez Gulf groundwater basin and exceeded water quality guidelines. The measured concentrations of Pb in most drilled wells were in the ranges 0.05–0.32 ppm (i.e. from a health-based guideline value of 0.05 ppm for drinking-water quality as recommended by World Health Organization (WHO 1984), to nearly seven times higher than this standard). Pb is more toxic, even at low concentrations and has no essential biological function but still accumulates in biomass and is freely transferred from one organism to another through the food chain (Mavropoulos et al. 2004). Pb reduces chlorophyll production and plant growth; increases superoxide dismutase (Gardea-Torresdey et al. 2005). For these reasons, Pb need to be monitored and their concentrations must be regulated.

From the limited information available, the monitoring process depends on the hypothesis that such increases in heavy metal concentrations represent an increase in

availability. As a consequence, some sites contain enough amounts of heavy metals such as Pb to reach toxic levels. Petroleum refining constitutes point sources due to hazardous spills, and refining is the major type of industries where heavy metal is usually associated with organic pollution (colloids). The anthropogenic origins of mixed pollutants have led to interest in their distribution and fate in the environment, mainly the marine environment. The following section discusses a probable scenario for validation of the origin, dispersion and fate of heavy metals in this area.

3.1 Structure Pattern, Intensive Use of Groundwater and Contamination Mixtures Mobilization Scenario

Since colloid-facilitated contaminant transport has gained broad acceptance, toxic metals are often associated with organic pollutants in polluted sites. Agreeing to Honeyman (1999), TPH (total petroleum hydrocarbon) represents the colloids that intermediate in the scavenging of metals in aqueous medium. Also, Snousy (2014) mentioned the relationship between Pb and Fe concentrations in this area and indicated that the high concentration of Fe (8923.91 mg/kg wet soil) plays an important role in scavenging of heavy metals such as Pb. This deduction agrees with Taillefert and Gaillard (2002). Abdel-Hamid et al. (2011) indicated that the sediments in northwestern Suez Gulf showed significantly high concentrations of Fe. Iron oxyhydroxides play a critical role in the fate and transport of potentially toxic metals such as Pb in natural systems due to their ubiquity and capacity to sequester metals (Hochella et al. 1999). This assumption is in line with the results of Burton et al. (2008) and references within, they indicated that coastal plains are underlain by soils that are hyper-acidic due to the oxidation of iron-sulfide minerals. Iron-sulfide oxidation in these soils (termed Coastal Lowland Acid-Sulfate Soils; CLASS) occurs when the previously waterlogged sulfidic soils are allowed to drain. Drainage may occur naturally (e.g. due to isostatic uplift), but is mostly due to land management intervention. Iron-sulfide oxidation associated acidification contributes to the release of iron, and trace metals such as Pb. This is geochemically important in reductive precipitation of Pb.

Pb becomes the repository for unprecedented volumes of toxic metals in metal-contaminated soils. Release of these elements from CLASS has caused widespread water quality degradation. Chaney et al. (1999) indicated that soil acidification increases solubility of Pb and other toxic metals and creates an additional environmental risk. Pb presented in soils is readily released into soil solution by heterogeneous precipitation at the contaminant/water interface in soils, with implications for the transport and distribution of Pb in the environment (Fig. 5). Leaching of Pb from the soil increases with increasing precipitation of the Fe that causes hyperacidity, thus Pb ultimately becomes a continuous source of the groundwater contamination. This point of view matches with the results of Gadd (2005) who mentioned that, precipitation of Fe is a significant process in highly contaminated soils.

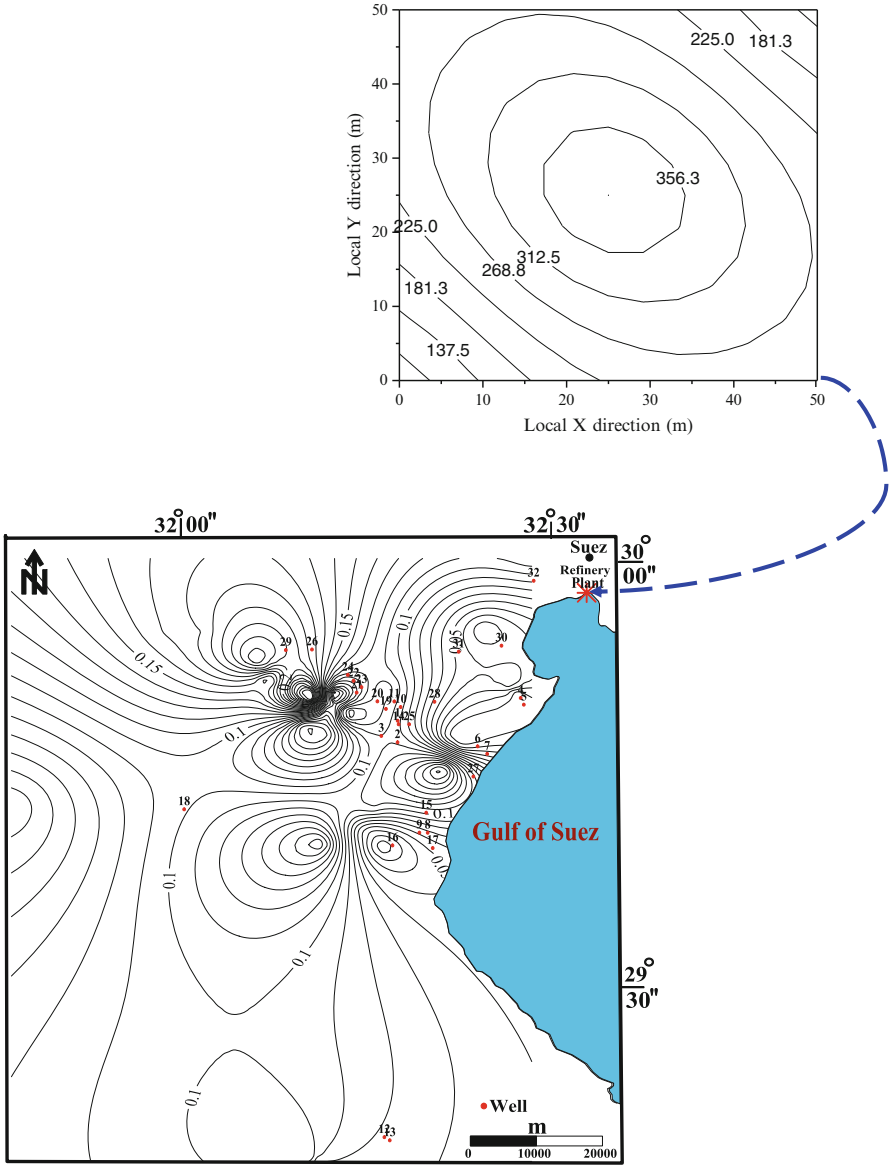

Fig. 5 and Fig. 6 Concentration map of Pb in soil (mg/kg), around the point 29° 57\ 33\\ N, 32° 30\ 40\\ E (industrial land) (Snousy 2014). Pb concentration (ppm) map in groundwater for 32 wells

In order to estimate the long-term impact, mobility, and prediction of its evolution in the pore water, the large scale distribution of Pb is shown in Fig. 6. That suggests a strong association of Pb in form of soluble metal complexes with anthropogenic organics, also humic substances that act as colloidal vehicles for heavy metals.

Humic substances (HS) are natural organic substances that are widely distributed throughout nature. They are commonly found in surface and subsurface water as well as in sediments and soils. As secondary products from the microbial decomposition of plant and animal remains, HS are organic polymeric composites that have no particular set of elemental composition or chemical structure (Yamashita et al. 2013). On the other hand, humic substances are operationally defined groups of natural organic matter that contain mixtures of chemically diverse organic components, presenting a spectrum of reactive functional groups ranging from weakly binding carboxyl and phenol to strongly binding amino and thiol groups (Tsang et al. 2013). Based on solubility characteristics, HS have been classified into three main fractions: fulvic acid, which is soluble in both acids and bases; humic acid (HA), that is soluble in bases but insoluble in acids (pH <2); and humin, that seldom dissolves in either acids or bases. According to this definition HA is regarded as a substance that drastically transforms itself in response to environmental conditions. It has generally been recognized that HA easily associates with other chemicals in the natural environment and significantly influences the chemical speciation and/or transport of other chemicals such as metals, hydrophobic organic pollutants and others. HA also alters the stability of colloidal particles, results in an enhancement of their transport. HA is negatively charged as a result of dissociation of its acidic functional groups; therefore, it has polyelectrolyte properties including protonation behavior, electrostatic potential and binding affinity to metal ions (Yamashita et al. 2013).

It has been also shown that dissolved organic matter facilitated metal transport, controlled metal solubility and speciation by the formation of soluble complexes and by adsorption onto the mineral phase. In addition, lignite-derived humic substances were found capable of binding a diverse range of heavy metals (Tsang et al. 2013). These studies have been effectively elucidating the mechanism of colloid-facilitated transport, in which colloids serve as carriers of other chemicals in the natural environment.

Acidic waters have the highest trace-element concentration such as Pb, and are rich in humic substances. The coastal sites in the Suez Gulf are organic-rich environment, assuming that the organic matter in the soil is humic acid-like (act as organic ligands) as mentioned by Cancès et al. (2003). Pb is a strongly adsorbed element with an intermediate affinity for dissolved organic matter. Carboxyl, phenolic, alcohol, and carbonyl functional groups in the humic substances react with Pb, forming metal–humate complexes (metal chelation) and stabilizing Pb Nannipieri (1994), Dick (1997) and Pascual et al. (1998). Also, Humic substances represent 70–90 % of dissolved organic carbon (DOC) in wetland areas (Thurman 1985). Despite their heterogeneity and complexity, humic substances are characterized by similar functional groups (carboxyls, quinones, phenolic OH groups) and the presence of aliphatic and aromatic components (Stevenson 1994). Metal binding in natural environments is, however, mainly related to carboxylic and phenolic groups (Perdue et al. 1984). Because of the high affinity of Pb for organic matter content (Sauvé et al. 2000), Pb is stabilized and widely dispersed because of high pumping or withdrawal rate. Groundwater is being excessively pumped in the

coastal zone through many wells that are dug by the citizens and investors. It is known that about 90 % of its current water consumption is extracted from groundwater with intensive extraction rate about 3000 m^3/day.

There is no advanced waste treatment employed to remove contaminants in northwestern Suez Gulf, and contaminant concentrations rapidly increased because of growing waste discharges. These activities have resulted in fluctuation of suspended and stabilized Pb in the whole groundwater basin. Consequently, leaching of Pb from water-sediments (from sediment-bound Pb) that contains elevated concentrations of Pb, constitutes a high pollution risk for adjacent areas because of the transportation of the Pb by water. Structural patterns (see Fig. 6) also support this point of view. The other possibility of Pb dispersion in this scenario is that the industrial effluents containing heavy metals also pollute soil and groundwater through seepage or by discharging them into Suez Gulf water nearby. Likewise, waters from open and bore wells (especially coastal wells) also become polluted with Pb because of pollution from industrial effluents nearby. These phenomena influence the aquifer's ecosystem functioning as a continuous source of water and eventually deteriorate its water quality. In addition, the coastal zone is liable to seawater intrusion or sea water encroachment. Figure 7 summarizes Pb mobilization pattern according to these scenarios.

Leachability of Pb and other organic and inorganic pollution increased during a long period of soil pollination and erosion, and intensive periods of sea water regressions and transgressions. Soil organic matter is thus a complex mixture of heterogeneous organic compounds (including sugar, starch, protein, carbohydrates, lignin, waxes, resins, and organic acids) derived from plants, microorganisms and animal residues that are formed through the decomposition, synthetic, and polymerization reactions. Soil organic matter here comes mainly from highly carbonized

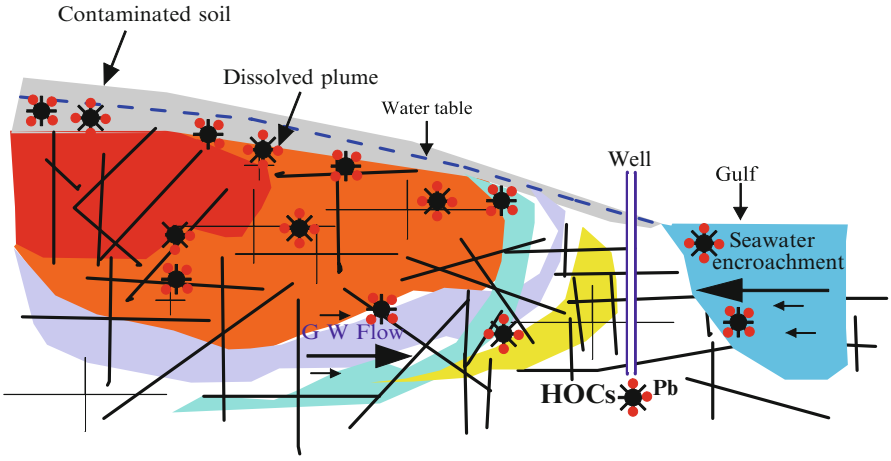

Fig. 7 A sectional view of the investigated area and schematic representation of possible conceptual scenarios for source zone and dispersion dissolved containment plume in a fractured media (not drawn to scale)

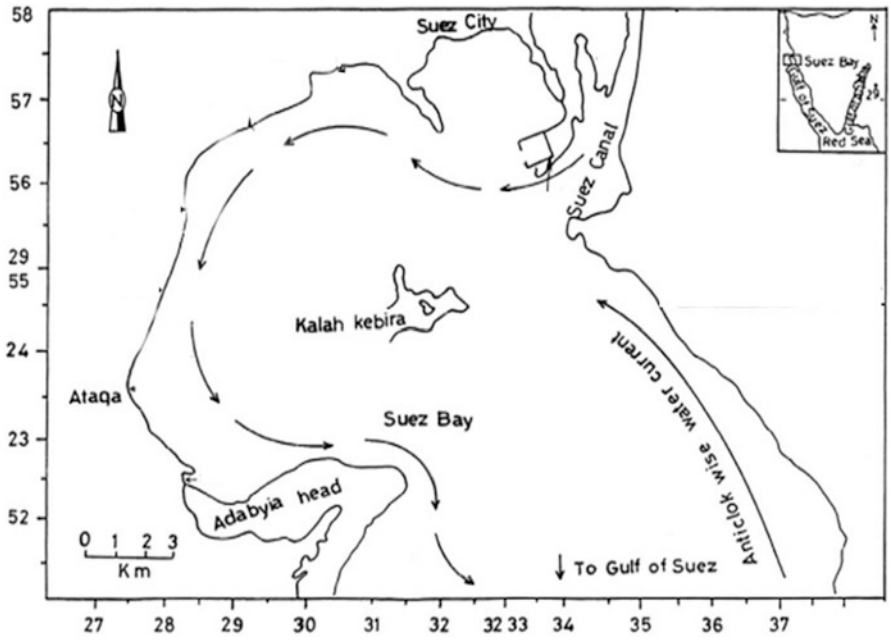

Fig. 8 Map of the Suez Bay showing the direction of water movement that causes transportation of pollutants (El-Moselhy and Gabal 2004; Belal and Ghobashy 2012)

compounds related to crude oil and its derivatives (e.g., charcoal, graphite, coal), and from natural marine products (natural organic matter) coming from Gulf water. Soil containing toxic metals, e.g. Pb, from long-term inputs of contaminants contains less biomass and altered microbial functionality. Soil humic substances are the stable part of decomposing organic matter in nature; carboxyl groups play an important role in stabilizing Pb in the humic acids (McKnight et al. 2001). Pb chelated by humic acids is more effective since humic acids provide more binding sites because of their larger molecules and more complex nature (Lobartini et al. 1994). Also, humic substances have more strongly acidic groups (Hayes 1991).

Suez Gulf is a eutrophic region (Hamed 1992), the northern part (Suez Bay) is characterized by anticlockwise water circulation (Meshal 1970; Soliman 1996; El-Moselhy and Gabal 2004) as shown in Fig. 8. The Suez Bay ecosystem is suffering of adverse effects of sewage discharge as well as industrial wastes from various activities such as oil refineries. As a result of such industrialization, Gulf coastal areas are increasingly subjected to various pollutions with subsequent threat of human health. Belal and Ghobashy (2012) stated that petroleum hydrocarbons pollution produced from oil production companies possibly are the reason of the absence of settling organisms in some localities northwest Suez Gulf, where these refineries discharge huge amounts of marine water contaminated with oil (16×10^3 m^3 h^{-1}) (Said 1992). Consequently sediments are polluted by a great amount of petroleum hydrocarbons that reach to 359.6 μg g^{-1} (Belal 1995).

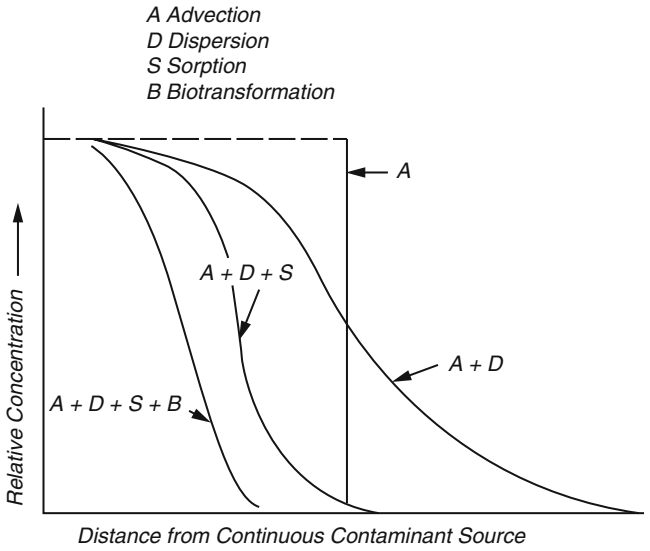

Fig. 9 The influence of natural processes on levels of contaminants from continuous and release sources (Keely et al. 1986)

Hamed and Said (2000) indicated that the anticlockwise circulation of water of the northern part of the Gulf of Suez brings the petroleum contaminants to some locations in the south. As with flow system characterization, contamination characterization begins with understanding the additional processes that affect transport of reactive contaminant. Dissolved contaminants in saturated porous media are controlled by adsorption, desorption, chemical reactions, and biological transformation. The processes of adsorption-desorption, chemical reactions, and biological transformation play important roles in controlling the migration rate as well as concentration distributions. These processes tend to retard the rate of contaminant migration and act as mechanisms to reduce concentrations. Because of their effects, the plume of a reactive contaminant expands and the concentration changes slower than those of an equivalent nonreactive contaminant as shown in Fig. 9. As mentioned previously Belal and Ghobashy (2012) stated that the absence of settling organisms in some localities northwest Gulf of Suez is due to petroleum hydrocarbons pollution and presence of toxic hydrocarbons. The role of natural biodegradation and biotransformation in the retardation of contaminant plume dispersion can be neglected. Likewise, sorption hasn't a strong role in decreasing neither hydrocarbon nor combined heavy metals, because of a long temporal and continuous release of these contaminants over sediments and adjacent Gulf water body. This is the reason for increasing transverse dispersion and expanding of the contaminant plume (Belal and Ghobashy 2012).

The mobility of contaminants, such as hydrocarbons and heavy metals, in the saturated subsurface zone is dependent on their distribution between the immobile solid phase and the mobile aqueous phase. The presence and fate of contaminants in

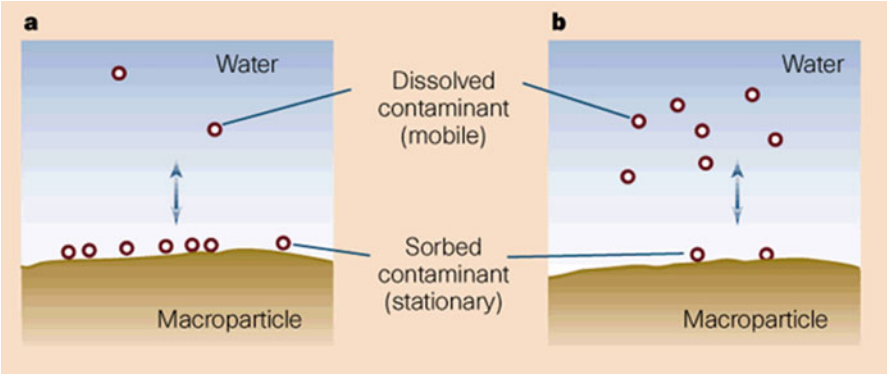

Fig. 10 Contaminant transport in a simple two-phase groundwater system. (**a**) High sorption and therefore low contaminant solubility; (**b**) low sorption, high contaminant solubility. Macroparticles are the stationary components of a groundwater aquifer and include clays, metal oxides (Honeyman 1999)

soils are of significant health and environmental concern because of potential high toxicity to humans and the serious effects that may result from direct and indirect exposure of ecosystems to metals. Many contaminants readily sorb to immobile aquifer media and therefore are considered to be virtually immobile in the subsurface, thus presenting little danger to groundwater supplies. Therefore, the predictions of contaminant transport are based on two phase equilibrium adsorption models (a dissolved phase and a sorbed immobile phase). The greater the extent to which a contaminant partitions onto the immobile phase, the slower is its average transport velocity in the groundwater shown in Fig. 10. This is the pattern of *contaminant transport in the absence of mobile colloidal fines in subsurface environment* (Sen and Khilar 2006).

Sen and Khilar (2006) mentioned that the unexpected appearance of low-solubility contaminants, some distance from known source or sooner than would be expect from their solubility, led to examination of the possible involvement of nonaqueous, *mobile colloids in contaminant transport*. The presence of mobile colloidal fines (organic hydrocarbons) explains such observations and lead to the conceptual model thinking of three phases in contaminant transport models i.e. mobile liquid phase, mobile colloidal phase (hydrocarbons, and heavy metals) and the immobile solid phase (Fig. 11). This contaminant transport has been known as "*colloid-facilitated contaminant transport*" (i.e., organic macromolecules and inorganic microparticles) which affect the transport of natural metals and anthropogenic contaminants in *hydrologic environments*.

Also, changes in environmental conditions (redox potential, pH, ionic strength or concentration of anions/cations, presence of organic ligands, concentration of competitive metals, soil/solution ratio) affect transport of heavy metals in the subsurface. Non-linear reversible sorption of metals to the soil matrix is a key process, resulting in partitioning of the metal mass between an adsorbed and a dissolved phase.

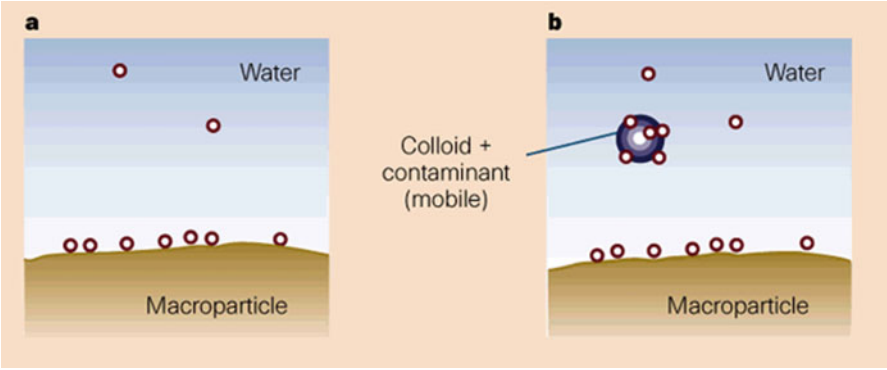

Fig. 11 Comparison of generalized two and three-phase groundwater system. (**a**) Two phase; (**b**) three phase. The third phase in (**b**) is a colloidal fines or microparticle, shown here with contaminant molecules sorbed to it, thus making them mobile (Honeyman 1999)

Moreover, the adsorption properties of a given metal in the presence of other metal (s) and/or organic ligands are often different from those of the individual metal as mentioned by Janetti et al. (2013). This occurs because of the presence of a finite numbers of adsorption sites, and a strong competition among sorptive ions for the same sites. These dynamics strongly affect the transport (mobilization and migration) of metals in porous media (Voegelin et al. 2001; Antoniadis et al. 2007). The experiments have focused on competitive sorption of metals in batch tests. These studies provide incomplete information (because it might lead to inadequate estimates of the space-time evolution of concentration in a flowing system) and do not fully capture solute transport dynamics, as analysis of metal retention from batch tests results in biased estimates as compared to flow-through experiments under competitive conditions (Seo et al. 2008; Rubin et al. 2012). One should note that batch experiments represent a single point only or a line of a multidimensional geochemical system (Jakob et al. 2009). A common mechanism of metal retention is the binding of cations with negatively charged surfaces (e.g., clay particles or organic matter that are present in the soil). These links, known as non-specific sorption, are relatively weak and broken by ions having higher affinity with the soil surface. Cation exchange in groundwater is the dominant surface reactions that occur in natural aquifers (Janetti et al. 2013).

Finally, as industrial activities in the northern Suez Gulf continue public and governmental efforts must be unified to protect this environment. The understanding of factors that govern the mobility of this set of chemical species (hydrocarbons, PAHs and Pb) is essential to assess possible risks to ecosystems quality in the study area.

These factors include a continuous release of mixed contaminants heavily seepage resulting from industrial activities, anticlockwise water circulation in Suez Bay, geology specially structure pattern, high pumping rate and intensive use of groundwater from drilled wells in this area. Also, the short-distance pathway needed for these migrating mixed contaminants to be released into the groundwater zone. This phenomenon efficiently minimizes the effect of natural attenuation of

these contaminants. This mechanism is expected to cause the occasional release of considerable amounts of contaminants, including hydrophobic organic contaminants (HOCs) and heavy metals (especially Pb), into groundwater in the vicinity of the northern Suez Gulf. However, the HOCs and heavy metals present in the contaminated soils can be remobilized from the soil as a result of shallow groundwater levels and infiltrating water. Thus, they become a long-term contamination source for the underlying aquifer and its water quality will be affected by the plume migration of leachate. All of these factors are important for soil, surface and ground waters pollution scenarios. These processes and requirements provide a broad view of the factors affecting contaminant transport from a site. Such understanding is depending on geoenvironmental and geochemical conditions to evaluate the processes that affect the transport of contaminants, including predictions regarding contaminants persistence and mobility. The discrepancy of the data could be attributed to the lack of extended data sets of contaminants pollution source, monitoring soil and water systems at large scales.

4 Summary and Conclusion

The northwestern Suez Gulf region is a strategic area in Egypt. The successive governments have established huge projects to achieve the development goals in this area. These different activities in this area depend mainly on the groundwater that is pumped intensively from different water bearing formations or aquifers. The pollution reduction and environmental quality control activities are important ingredients of any economic development program. This review compiles the previous studies from the 1980s up to 2015, studies that are concerned with estimating the concentrations of different pollutants in various ecosystems in the northwestern Suez Gulf region. The geomorphological/vulnerability assessment shows that the greatest part of the Egyptian Red Sea coastal environments are vulnerable, i.e. they possess high biological susceptibility to immediate and long term oil spill damage resulting in occurrence of different pollutants, particularly in the northern Suez Gulf. As long as industrial activities in the northern Suez Gulf continue to increase, public and governmental efforts must be united to protect these complex ecological systems through improvement of the wide range monitoring programs. This issue has not been extensively surveyed before, and this review, gives specific directions for future monitoring and remediation strategies in this region. This study helps decision-makers to assess strategies for preventing pollution and detecting the contaminated spots, and also to establish criteria, protocols and monitoring plans for integrated management strategies related to the public health point of view, leading to sustainable development policy.

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